Regulation of Ovarian Function by the Germ Cell Specific DAZL Gene

Yvonne A. R. Brown
BSc (Hons), University of Aberdeen
MSc by Research, University of Edinburgh

Medical Research Council
Human Reproductive Sciences Unit
Queen's Medical Research Institute
47 Little France Crescent
Edinburgh, EH16 4TJ
United Kingdom

Thesis submitted to the University of Edinburgh for the Degree of Doctor of Philosophy

September 2008

Declaration

The studies undertaken in this thesis were the unaided work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for another degree or qualification.

Yvonne A. R. Brown

September 2008

Acknowledgements

Throughout my PhD I have received a vast amount of training, encouragement, support and friendship, and there are a number of special people I would like to acknowledge and thank.

My first and biggest thank you goes to Alan McNeilly. I could not have wished for a better supervisor. Your immense support and enthusiasm for reproductive biology has kept me enthralled throughout my work. Your passion for science has enabled me not only to produce this thesis but to realise why I started this in the first place and you have given me great guidance for the future.

Next is the McNeilly group which has been one of great support, kindness and fun! Judy McNeilly, you have been a fantastic friend and listener over the past few years, always full of kind words and good advice, thank you. Also thank you to Linda Nicol, you have been so helpful with all aspects of the lab and in particular with molecular biology and not to mention answering endless questions! Thank you also to Mhairi Laird and Sarah Henderson for all your help, company and lab chat (and of course the dancing!!). For their technical advice and support I would like to thank the always helpful Histology team, Rosey Bayne, and to Mark Fisken for all his help with the mice.

Fantastic friends, both past and present, have played a dominant role in making the past few years roll by, friendships and memories are ones to keep for life so thank you. But most notably thank you Hayley for making life so interesting, fun and truly being the best friend you could ask for.

Huge thanks to my family, Mum, Dad, Veronica, Gareth, and Grandparents for all your constant love, support and encouragement along the way.

Lastly, my most loving thanks goes to Colin. You have supported me through every step of this PhD and have always kept me positive by believing in me. The laughter, the tears and the tantrums have hopefully made it all worth while!!

Yvonne
2008

Abstract

The RNA binding protein DAZL (Deleted in Azoospermia) is essential for germ cell survival and subsequent fertility. The transgenic mouse DAZL model has confirmed that knockout (KO) females are infertile as a direct consequence of complete postnatal oocyte ablation. Interestingly, the heterozygous (Het) DAZL females have increased fertility giving rise to significantly more viable offspring, accompanied by significantly reduced plasma FSH and increased inhibin B compared to levels observed in the wildtype (Wt) females. Recent studies to identify putative DAZL mRNA targets suggest that DAZL may have multiple functions and mRNA targets throughout germ cell development. However, how this protein functions within the oocyte and how functional copy number gives rise to increased fertility remains to be fully elucidated.

The studies in this thesis sought to identify putative DAZL mRNA targets in addition to molecular mechanisms which may be either affected direct or indirectly as a result of the functional copy number of DAZL (Wt or Het) within the oocyte or follicular unit. Oocytes from Wt and Het were evaluated for their expression of selected oocyte genes and comparative analysis suggests that oocyte gene expression is significantly altered between the genotypes. Genes of interest include Oosp1 and H1foo, both of which are down-regulated in mRNA expression in Het d21 oocytes and d10 ovaries compared to the Wt. Furthermore, an *in silico* bioinformatics approach was utilised to identify putative DAZL mRNA targets using a consensus DAZL binding sequence. One candidate target, PDCD4, previously identified as a tumour suppressor gene was selected for further investigation. Despite PDCD4 mRNA and protein being highly expressed within the ovary, no difference in mRNA levels between Het and Wt was observed. However, although not ruling out the possibility of being a DAZL target we now have evidence that PDCD4 can function within the steroidogenic cells of the corpus luteum in relation to functional luteolysis.

Indirect actions of DAZL upon local regulation and response of follicle growth in culture were evaluated to investigate follicles at the gonadotrophin dependent stage of growth. Individual follicles from Wt and Het d21 mice were cultured in the presence of FSH at 1iu, 0.5iu, 0.1iu and 0.01iu for a six day period. Final follicle size/morphology did not differ between genotypes at 1iu, 0.5iu and 0.1iu of FSH, but by d3 at 0.01iu FSH growth of Wt follicles was significantly ($P<0.001$) perturbed compared to the Het. Despite no difference in final size between 1iu, 0.5iu, 0.1iu FSH treatments, mRNA analysis of individual follicles demonstrated a significant up-regulation of FSH receptor ($P<0.05$), aromatase ($P<0.05$) and inhibin βA ($P<0.01$) and a significant down-regulation in inhibin βB ($P<0.01$) expression in the Het follicles compared to the Wt, suggesting an increase in follicle maturity, sensitivity and hence suitability for selection as viable pre-ovulatory follicles. Furthermore, atresia rates from cultured follicles were significantly lower ($P<0.05$ (1iu, 0.1iu FSH); $P<0.01$(0.01iu FSH)) in the Het compared to the Wt.

These studies provide strong evidence that multiple mechanisms within the oocyte/follicle are directly and indirectly affected as a result of functional copy number of DAZL. Although direct *in vivo* targets remain to be identified it is clear that DAZL protein potentially targets multiple mRNAs at different stages of development, pre-programming the oocyte to increase the sensitivity of follicle and/or the functioning within a transcription complex regulating development. In conclusion, the beneficial consequences of increased fertility in the Het females is accompanied by a possible acceleration in oocyte and follicle maturation, an increased sensitivity to FSH *in vitro* with evidence of advanced stages of growth and, a reduction in follicle atresia. These differences support the suggestion that DAZL is having systemic effects on the paracrine communication within the follicle unit between the oocyte and somatic cells altering regulation and subsequent selection, and affecting final ovulation rate and litter size.

Presentations relating to thesis

Oral Presentation, SRF Annual conference, Leeds University, Leeds, 2006:

"The Pro-Apoptotic Protein PDCD4 is a Potential Target for DAZL in Mouse Oocytes".

Oral Presentation, EggNet Edinburgh, April 2007:

"The influence of DAZL on Follicle Development".

Oral Presentation, SSR, San Antonio, Texas, 2007:

"Novel Expression of the Pro-apoptotic Factor PDCD4 in the Rodent Ovary".

Poster communication, SRF Annual conference, Edinburgh, 2008:

"Gene profiles during maturation of ovarian follicles from WT and Het DAZL KO mice".

Abbreviations

°C	Degrees Celsius
A	Adenine
Ab	Antibody
ABC	Avidin Biotin Complex
AMH	Anti-mullerian hormone
ANOVA	Analysis of variance
AR	Androgen receptor
AR KO	Androgen receptor knockout mouse
ArKO	Aromatase knockout mouse
BERKO	Oestrogen receptor beta knockout
BMP	Bone Morphogenetic Protein
bp	Base pair
BrdU	Bromo-2'-deoxyuridine
BSA	Bovone Serum Albumin
C	Cytosine
cDNA	Complementary deoxyribonucleic acid
CL	Corpus luteum
CO_2	Carbon dioxide
d	day
DA	Dalton
DAB	Diaminobenzidine
DAZL	Delected in Azoospermia
dH_2O	Distilled water
dNTP	Deoxynucleotide Triphosphate
e	Embryonic
ERKO	Oestrogen receptor alpha knockout
ERα	Oestrogen receptor alpha
ERβ	Oestrogen receptor beta
ES	Embryonic stem cells
Figla	Factor in the Germ line alpha
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
FST	Follistatin
g	Gram
G	Guanine
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
GnRH	Gonadotrophin releasing hormone
hCG	Human Chorionic Gonadotrophin
Het	Heterozygous
HPG	Hypothalamic pituitary gonadal

IgG	Immunoglobulin G
ip	Intra-peritoneal
IU	International unit
J	Joules
kg	kilogram
KitL	Kit Ligand
KO	Knockout
L	Litre
LH	Luteinising hormone
LHR	Luteinising hormone receptor
M	Molarity
m	Meters
MEM	Minimal essential medium
MFOs	Multi oocyte follicels
mg	milligram
$MgCl_2$	Magnesium chloride
Min	Minute
ml	millilitre
mm	millimeter
MMP2	Matrix metalloproteinases
mRNA	Message Ribonucleic Acid
n	nano
oFF	Ovine follicular fluid
OSE	Ovarian surface epithelium
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PGCs	Primordial germ cells
PMSG	Pregnant mare serum gonadotrophin
rpm	Revolutions per minute
SEM	Standard error of the mean
T	Thymine
TBS	Tris buffer saline
tfm	Testicular feminised
TGF β	Transforming growth factor beta
Tm	Melting temperature
UTR	Untranslated region
UV	Ultra Violet
V	Volts
v\v	Volume to volume
Wt	Wildtype
αβERKO	Oestrogen receptor alpha and beta knockout
µ	Micro

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Chapter 1: Literature review

Regulation of normal female reproductive function is principally governed by the finely coordinated actions of the gonadotrophin hormones which systemically regulate folliculogenesis. In turn folliculogenesis is a process which encompasses local steroidogenesis, gene regulation, cell cycle progression, cytodifferentiation and apoptosis (Richards et al., 1995). Oocyte expressed genes notably play an important role in producing vital regulatory molecules that directly affect fundamental physiological processes during folliculogenesis (Erickson and Shimasaki, 2000). Acknowledging the importance of these oocytes factors implies that any alterations or disruptions of these specific genes may lead to infertility and ovarian pathology (Erickson and Shimasaki, 2000). However, the oocyte does not function alone and it has long been established that the communication between it and the surrounding somatic cells of the ovarian follicle are necessary for appropriate functionality. From a historic perspective the identification of the physiological importance of somatic cell-oocyte interactions dates back to 1959, whereby it was reported that the after ovectomy spontaneous transformation of the large Graafian follicle into a corpus luteum occurred (Falck, 1959). From these observations, which have subsequently been confirmed by others (El-Fouly et al., 1970; Channing 1970; Hubbard and Erickson 1988; Vanderhyden et al., 1993), it can be concluded that the oocyte produces essential signals which subsequently suppress luteinisation and, upon removal, these signals cease to operate, hence onset of luteinisation. Moreover, it has now been acknowledged that this communication is a two-way mechanism requiring a plethora of essential mediators with alterations in control being responsible for many underlying disorders of female reproductive health. To understand and appreciate the complexity of this bi-directional control of the follicular unit we have to acknowledge the sophistication of the developmental systems involved, starting from embryonic development and ending with ovarian quiescence, whereby the oocytes pool is essentially depleted.

A finite, non-renewable pool of primordial follicles is believed to be present at the time of birth and if correct the supply of primordial follicles in postnatal life should

be depleted during follicle development, either by undergoing atresia or surviving to ovulation (Kerr et al., 2006). As a result of this predetermined supply of oocytes it is fundamentally important to ensure that the supply of oocytes is not wasted and that follicle development occurs efficiently. The cessation of the ovarian function occurs when the ovary becomes exhausted of germ cells, which is classified as menopause in human females. However recent evidence suggested that this may not be the case and oocyte renewability has been a target of biological investigation and subjected to recent debate. This original dogma, has been recently challenged by Tilly and colleagues (Johnson *et al*., 2004; 2005), who suggest that intra- and extra-ovarian germline stem cells can replenish oocytes and form new primordial follicles in adult life. However, this new concept has been hotly disputed between Tilly's laboratory and others within the ovarian field (Telfer *et al*., 2005; Powell, 2006; Faddy and Gosden, 2007; Tilly and Johnson, 2007). In support, a further study in mice shows that despite continued activation of follicles throughout ovarian cycles, there was little reduction in the overall number of follicles from birth up to 140 days of ages suggesting that follicle replenishment may be occurring in these mice (Kerr et al., 2006). However the authors do not propose the presence of germline stem cells as suggested by Tilly, and with no clear explanations of their result the debate continues.

Indisputably, reproducing is fundamental to the survival of mammals, therefore an understanding into the normal biological function within the ovary is essential before attempting to investigate ovarian abnormalities which persist in development and contribute to infertility. Many factors have been implicated as essential regulators in germ cell development and follicle organisation although the molecular underpinnings of what constitutes an oocyte of high quality are essentially unknown (Pan et al., 2005). Investigations into the delicate interplay between the molecular mechanisms and follicular productivity remain the focus of this thesis with emphasis on the functions of the germ cell specific gene DAZL and its contribution to functional follicular development. In the absence of DAZL oocytes fail to enter meiosis, and subsequently die prior to birth leaving the mice infertile (Ruggiu et al., 1997). Therefore, not only is the DAZL knockout (KO) mouse an invaluable

research tool aiding the investigations into the mechanisms by which DAZL protein affects oocyte maturation and hence fertility, it subsequently allows us to investigate the heterozygous (Het) phenotype which present with increased fertility. In order to establish why complete functional loss of DAZL is detrimental to fertility but reduced function as in the Hets increases the number of viable offspring, it is first imperative to consider how DAZL is affecting the development and regulation of the oocyte and consequently the follicular unit as a whole. Furthermore, attempting to identify targets of DAZL and pathways in which it may play regulatory roles is imperative to identifying the importance of this oocyte regulator. This review aims to deliver an overview of selected mechanisms which are of great importance in female reproductive development highlighting the need for a more cohesive understanding of the complexity of the molecular mechanisms involved.

1.1. Folliculogenesis

The development of the mammalian ovary is a complex process requiring the fine coordination of specifically defined events including embryogenesis, oogenesis and folliculogenesis.

Throughout reproductive development and function there are a number of germ cell fates. These include the loss in fetal life by apoptotic cell death, loss as a result of atresia at any stage of follicle development, or they can be recruited into the follicle cohort destined for dominance and subsequent ovulation. The ultimate success of the oocyte is fertilisation giving rise to a viable new generation. All these events require the appropriate coordination of many regulatory mechanisms relying on gene expression and protein production as selectively discussed in the following sections. Furthermore the ovary participates in extensive tissue remodelling required for follicular development, ovulation and corpus luteum (CL) formation. Conclusively the ovary is one of the most active endocrine organs both functionally and structurally during reproductive life in mammals.

1.1.1. Migration of germ cells

The journey of germ cell existence starts in fetal life with the primordial germ cells (PGCs) arising from the extraembryonic epiblast cell precursors located outside the embryo. In the mouse the founding population of PGCs is around 45 and can be visualised at embryonic day (e) 7.2 (Tam and Snow, 1981; Ginsburg et al., 1990). These PGCs migrate through the hindgut mesentery to the genital ridge which is formed from the mesoderm layer and differentiates into the mammalian gonad. During the process of migration the PGCs continue to proliferate and begin to aggregate with one another, with approximately 3000 cells detectable at e11 in the mouse. This proliferation continues within the genital ridge and by e13.5, around 25,000 germ cells are present (Monk and McLaren, 1981; Tam and Snow, 1981).

1.1.2. Germ cell cysts formation and breakdown

It is within the genital ridge that the PGCs combine with the somatic components of the gonad and form germ cell cysts (Bendel-Stenszel et al., 1998). These cysts are formed as a result of incomplete cytokinesis during cell division in the fetal mouse following germ cell migration into the genital ridge (Pepling and Spradling, 1998). Furthermore, from e13.5 after colonisation, the germ cells within the cysts enter meiosis. At the beginning of e17.5 on reaching the diplotene stage of the first meiotic prophase the oogonia arrest and are now recognised as oocytes (McLaren, 2000) (Figure 1.1).

After this episode of proliferation and migration up to 70% of germ cells within each cyst actually die between e13 and e16 (Baker, 1972; Coucouvanis et al., 1993; Ratts et al., 1995). In addition it has been postulated that this mass attrition is a process devised to eliminate germ cells harbouring chromosomal abnormalities or mitochondrial defects leaving only the best selection of germ cells for subsequent development (Baker, 1972; Krakauer and Mira, 1999; Morita and Tilly, 1999). In addition to this mass depletion, germ cell cysts also enter a developmentally programmed breakdown as a result of invasion by pre-granulosa cells prior to the formation of individually selected primordial follicles (Figure 1.1) (Pepling and Spradling, 2001; Pepling, 2006). This invasion and subsequent encasement of the

oocytes with pre-granulosa cells is a gonadotrophin independent event, occurring just after birth, involving cell specific (e.g. OCT4) and nonspecific (e.g. BMP4 and BMP8b) factors and although not fully understood at a molecular level it is clear that genetic regulatory control must be interacting between the oocyte and granulosa cells (Pepling, 2006). Furthermore, a role for nurse cell action and gradual breakdown of larger cysts into smaller cysts then to individual follicles has also been proposed (Pepling, 2006).

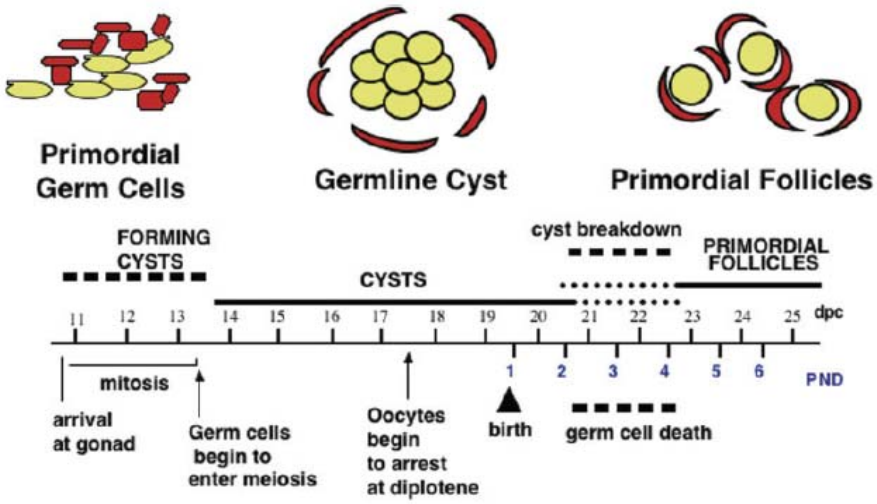

Figure 1.1: Summary of germ cell cyst formation, breakdown and primordial follicle formation in the mouse. The primordial germ cells arrive into the genital ridge at approximately e10, after which germ cell cysts form. From e13.5 the germ cells within the cysts progress through prophase 1 of meiosis arresting at the diplotene stage at the beginning of e17.5. In the neonatal ovary the germ cell cysts breakdown and pre-granulosa cells and basal lamina surround the oocytes forming primordial follicles (germ cells are represented by yellow and the somatic cells are in red) (Hirshfield, 1991; Barnett et al., 2006; Pepling, 2006).

1.1.3. Molecular control of germ cell cyst breakdown

The processes involving germ cell nest formation, breakdown and primordial follicle formation are regulated by a number of communicating factors (Pepling, 2006). Members of the TGFβ superfamily, specifically BMP15, GDF9 and inhibin α (McMullen et al., 2001; Yan et al., 2001b), the transcription factor Figla (Soyal et al., 2000), the notch signalling pathway (Hahn et al., 2005) in addition to steroids (Chen

et al., 2007) have all been identified as essential factors involved in these processes. Findings from a number of studies indicate that incomplete cystic breakdown occurs in conjunction with an increase in multi-oocyte follicles (MOFs) as a result of ablating BMP15 and GDF9 and over-expressing inhibin α (McMullen et al., 2001; Yan et al., 2001). These findings implicate the involvement of these factors directly in germ cell cyst breakdown. Furthermore, female mice lacking Figla show an absence of primordial follicle formation perinatally, this is accompanied by a deficit in oocytes (Soyal et al., 2000). Again these findings support the active role for Figla in the regulation of initial formation of primordial follicles playing an ,active role in modulating germ cell survival (Soyal et al., 2000). Furthermore, Figla is the transcription factor for the zona pellucida genes (ZP1, ZP2 and ZP3) (Liang et al., 1997) and mutations in these genes confirm that they too are crucial for successful follicle formation (Liu et al., 1996; Rankin et al., 1996; Rankin et al., 1999; Rankin et al., 2001).

Recent studies have also shown that oestrogen, progesterone and testosterone signalling are the primary signals required to initiate oocyte nest breakdown and follicle assembly. In support, on treating rodents neonatally with natural oestrogens, compounds with oestrogen-like activities, testosterone or progesterone all resulted in an increase in MOFs, suggesting incomplete nest breakdown (Iguchi et al., 1986; 1988; 1990; 2001; Iguchi and Takasugi 1986; Suzuki et al., 2002; Kezele and Skinner, 2003). However, it is not known whether the effects of progesterone and testosterone are direct or indirect due to there ability to convert to oestrogen (Kezele and Skinner, 2003).

Evidence is now emerging about the function of a host of critical factors associated with primordial follicle development, as described above, and further elucidation into the processes involved in the establishment of the primordial follicle pool will provide a better understanding of oocyte growth and development. The role of the oocyte and further follicular development will now be discussed.

1.1.4. Oocyte growth

The mouse oocyte takes approximately 2 weeks to reach full size and a further week to reach pre-ovulatory maturity (Bachvarova, 1985). During this growth phase the diameter of the mouse oocytes, which are arrested in diplotene stage of the first meiotic prophase, increases substantially from 10-15μm in the primordial resting follicle to approximately 80μm in the pre-ovulatory antral follicle (Gosden and Bownes, 1995) during which both meiotic and developmental competence are acquired. This remarkable enlargement of the oocyte occurs as growth is not delayed or interrupted by cell division and by the process of cytoplasmic cleavage. Moreover this substantial growth of the oocyte is a reflection into its maturity with an increase in both oocyte diameter and volume. Throughout oogenesis there are timely regulated periods of high transcriptional and translational activity, that alternate with phases of relatively low metabolic activity. However, in concordance with this increase in oocyte volume the activity of the growing oocyte is associated with the accumulation of RNA transcripts and the synthesis of proteins from both nuclear and mitochondrial transcripts. These products of oocyte activity are essential components required for the differentiation and maturation of the oocyte itself (De La Fuente and Eppig, 2001) and notably a number of oocyte derived proteins interact with the encasing granulosa cells, these are later discussed in detail. Furthermore these oocyte derived factors play important roles later in the activation of the embryonic genome and embryonic development (Amleh and Dean, 2002).

It has become increasingly apparent that genetic programming within the oocyte plays an important role in orchestrating the multiple events required for successful folliculogenesis, fertilisation and early development (Dean, 2002). To further appreciate oocyte growth it is probably more reasonable to observe its function as part of a developmental follicle unit rather than an individual cell. In discussing the roles of the oocyte in folliculogenesis it is apparent that oocyte growth is contributing to not only self survival and development but is involved in a network of highly regulated events.

1.2. Follicle development

The dynamic control of ovarian function is coordinated by a plethora of highly regulated control mechanisms. Major players involved in folliculogenesis are undoubtedly the gonadotrophins, however, these factors do not function alone and many control systems actively interact. The process of folliculogenesis involves small oocytes surrounded by a single layer of granulosa cells undergoing extensive morphological changes to reach a large meiotically competent oocyte surrounded by a multiple interconnecting granulosa cell layer further encased by a somatic thecal layer (Figure 1.2). This substantial growth can be divided into two phases, with the initial stages being predominantly gonadotrophin independent, and there is accumulating evidence that intra-ovarian paracrine signalling plays an important role (Kol and Adashi, 1995). The second growth phase of folliculogenesis is significantly regulated by the circulating gonadotrophins, follicle stimulating hormone (FSH) and luteinising hormone (LH). Again intra-ovarian factors have been implicated. Although the growth phases have been classified into gonadotrophin independent and gonadotrophin dependent the developmental stages of the actual ovarian follicles are often classified with reference to their morphology. More recently it has been recognised that the functional physiological state is a more accurate means of follicle classification (Hirshfield 1995; Scaramuzzi et al., 1993; Findlay et al., 2002). Using this physiological status, four classes of follicular growth have been identified based upon their individual dependency and sensitivity to gonadotrophins. Follicles can therefore be accurately classified as quiescent (primordial), committed to growth (preantral and antral), ovulatory or atretic.

It is now recognised that oogenesis and folliculogenesis are highly coordinated coupled processes and no follicle would be formed in the absence of an oocyte with the oocyte being regulated by its neighbouring granulosa cells. This intracellular communication between the oocyte and granulosa cells is essential for normal follicular differentiation and oocyte development. Events regulating the bi-directional communication between oocytes and somatic cells results in the granulosa cells influencing oocyte development, growth, meiotic arrest and transcriptional function. In addition, the processes occurring within granulosa cells

which are controlled by oocytes include proliferation, differentiation, follicle formation, cumulus expansion, steroidogenesis and ovulation (Eppig, 2001). These complex cell-to-cell interactions coordinate the development of ovarian follicles, from primordial through to antral stages, with the ultimate objective being ovulation and successful fertilization. However, the formation and control of these interactions and contribution of each individual cell type to the overall functioning of the follicle are poorly understood. Only a selection of genes having been investigated in depth and are known to be essential control factors (Acevedo and Smith, 2005; Sugiura et al., 2005).

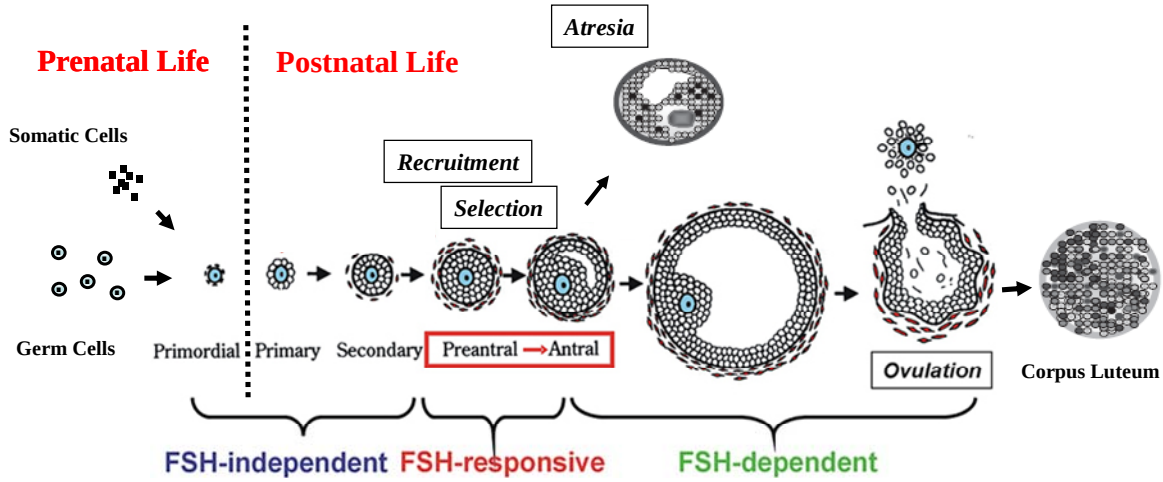

Figure 1.2: Schematic diagram of follicle developmental, survival, ovulation and atresia, showing prenatal primordial and postnatal follicle maturation. From the antral stage of follicle growth follicle become gonadotrophin dependent and a decision is made whether the can withstand the reduced threshold of the FSH decline and proceed to the pre-ovulatory stage or become atretic (Adapted from Barnett et al., 2006; Craig et al., 2007).

An example of how important this bi-directional communication is has been demonstrated by recent evidence which has shown that mouse oocytes control glycolysis in granulosa cells by regulating expression levels of genes encoding essential enzymes. The oocytes control the intercellular metabolic cooperative communication between cumulus cells and oocytes, which is required for energy production by granulosa cells essential for oocyte and follicles development (Sugiura et al., 2005).

Furthermore, oocytes and granulosa cells have the capability of being mutually independent in their survival and normal development despite their dual requirement for follicle formation. These independent characteristic substantially aid the practical implication of using these individual components for experimental culture manipulation and monitoring (Gosden and Boland, 1992).

In addition to providing structural support the theca cells of the follicle aid the establishment of mesenchymal epithelial cell-cell paracrine interactions between the granulosa cells and theca cells, which is essential for subsequent follicular development (Skinner, 1990; Parrott et al., 1994). Recruitment of theca cells from the surrounding somatic tissue to the periphery of the follicle occurs at later developmental stages of folliculogenesis. Intra-ovarian factors have also been recognised which may contribute to the coordination of the recruitment and regulation of the thecal cell function and Kit Ligand (KitL) has been described and identified as a key requirement in theca cell organisation (Parrott and Skinner, 2000). It has also been proposed that in addition to granulosa cell-theca cell interactions, oocyte-theca interaction occur. A study using rats has illustrated this oocyte-theca interaction, and demonstrates that the oocyte derived factor GDF9 can stimulate both basal and LH-stimulated theca interstitial cell androgen biosynthesis (Solovyeva et al., 2000).

Granulosa cells of pre-antral ovarian follicles differentiate into two distinct spatial and functional populations during advanced follicle transition. The cumulus granulosa cells are associated with the oocyte, and the mural granulosa cells surround and line the follicle wall, these two populations do have similarities but there are differences in both transcripts and proteins produced (Latham et al., 1999; 2004). For instance Kit Ligand mRNA which encodes KitL, previously mentioned to be a theca cell organiser, is increased in pre-antral granulosa cells and mural granulosa cells of antral follicles compared to KitL mRNA expression in the cumulus cells (Joyce et al., 1999). Furthermore, LH receptor (LHR) expression levels are also increased in the mural granulosa cells compared to that of the cumulus cells or pre-antral granulosa cells (Camp et al., 1991; Peng et al., 1991). These differences in

gene expression suggest alternative functions for these two sub population of granulosa cells in follicle and oocyte development, with the cumulus cells specialising in oocyte support and metabolism (Haghighat and Van Winkle, 1990).

The events surrounding the process of folliculogenesis include the acquisition of FSH receptors (FSHR) on the proliferating granulosa cells which is governed by influences imposed by both oestrogen and FSH. As a consequence of the presence of an elevated number of FSHRs, the granulosa cells of the small antral follicle are able to respond to FSH. FSH increases cyclic AMP accumulation, activation of the aromatase system, and induces LHR, which subsequently permits the granulosa cells to respond to LH. The circulating serum levels of LH increase during the preovulatory LH surge, and consequently a number of events occur: firstly resumption of oocyte meiosis, germinal vesicle breakdown releasing the first polar body and progression into metaphase stage of meiosis II where the oocyte arrests again until fertilisation (Greenwald and Roy, 1994), transformation of the steroid enzyme complex from oestrogen to progesterone secretion, follicular rupture, and lastly the formation of the corpus luteum.

The following sections of this chapter focus on the cyclic events surrounding folliculogenesis including the importance of recruitment and selection, the formation of mature follicles encompassing antrum formation and cumulus expansion and finally ovulation and corpus luteum formation.

1.2.1. Initial and cyclic recruitment

Recruitment and selection of primordial follicles is fundamentally important throughout the duration of female reproductive life. Only a select number of follicles ultimately achieve dominant follicles status developing to the advanced pre-ovulatory stages with the remaining subordinate follicles from the initial growing cohort becoming atretic (Hirshfield, 1992; Hsueh et al., 1994; Baker et al., 2001). It is believed that follicles leave the primordial pool in an ordered sequence and become irreversibly committed to growth. There is recent evidence (Durlingar et al., 1999; 2002) suggesting that AMH may play an important role in the initial recruitment of follicles from the primordial pool. More primordial follicles are recruited in AMH null mice compared to the occurrence of primordial follicle recruitment in wild-type mice, with the heterozygous mice taking an in-between position (Durlingar et al., 1999, 2002), thus suggesting a dose dependent inhibitory effect of AMH on follicle recruitment. However the rate at which these events take place and how additional signalling mechanisms are involved remains to be fully investigated (McGee and Hsueh, 2000) (Figure 1.3).

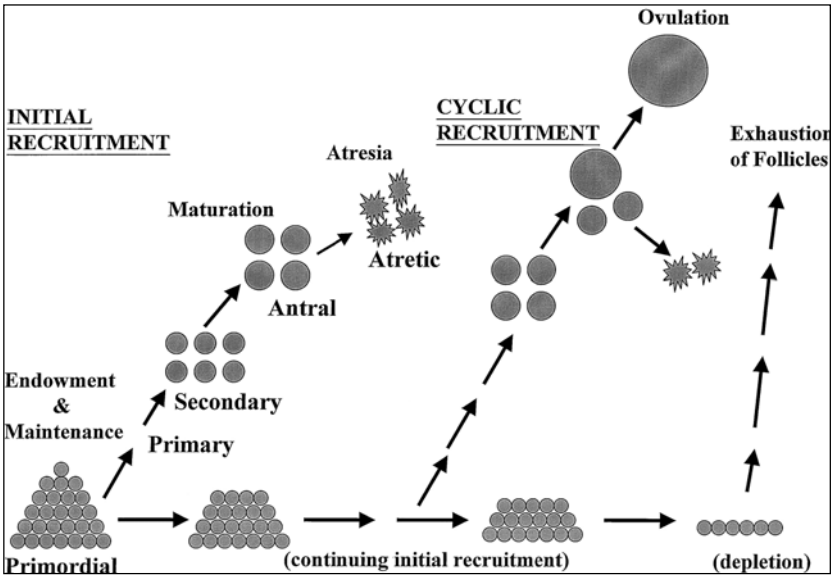

Figure 1.3: Life history of ovarian follicles; endowment and maintenance, initial recruitment, maturation, atresia or cyclic recruitment, ovulation and exhaustion. Initial recruitment is from the primordial resting pool, follicles develop through the primary stages to the secondary stages (gonadotrophin independent). At the antral stage unless responsive to gonadotrophins follicles become atretic. Eventual depletion of the ovarian follicle reserve occurs (McGee and Hsueh, 2000).

The major contributing factor influencing ovarian follicle selection for dominance is the decline of circulating FSH (Hirshfield 1991; Hsueh et al., 1994; Chun et al., 1996), which is brought about by the feedback control of the HPG axis by the production of ovarian oestradiol and inhibin (Figure 1.4). FSH is one of two major survival hormones essential for the prevention of programmed demise of growing early antral follicles. In addition FSH also plays a role in the final differentiation of granulosa cells which is accompanied by an increase in local production of oestradiol (Johnson and Everitt, 2007) and inhibin (Michal et al., 1993; Hayes et al., 1998) in preparation for follicular ovulation (Hsueh et al., 1994). These locally produced factors subsequently feedback to the pituitary resulting in a decline in plasma FSH concentrations to a level insufficient to maintain the growth of the subordinate less advanced follicles (Brown, 1978; McNeilly, 1988; Chappel and Howles, 1991). The dominant follicles survive this declining plasma FSH by virtue of their enhanced responsiveness to gonadotrophins in addition to transferring their dependence to LH (Webb and England, 1982; Ireland and Roche, 1983). Even though the granulosa cells acquired large numbers of LH receptors, they are prevented from luteinizing prematurely by factors in follicular fluid, including oestrogen. Furthermore as previously mentioned the oocyte has also been suggested to secrete factors which prevent luteinisation (El-Fouly et al., 1970; Channing 1970; Hubbard and Erickson 1988; Vanderhyden et al., 1993).

Despite the well documented mechanism for follicle dominance an additional regulatory mechanism has been suggested after experimentation using co-cultures of intact follicles. The results from these studies suggest that the contact communication between follicles of equivalent stages of growth is an important regulator in maintaining dominance. Furthermore it has been demonstrated that both endocrine and intra-ovarian factors from dominant follicles can promote direct effects on subordinate follicles, suppressing their development (Baker and Spears, 1999; Baker et al., 2001).

1.2.2. Follicle maturation, ovulation and corpora lutea formation

The transition from a secondary follicle to an antral follicle is associated with the formation of a fluid filled antral cavity with the initial appearance of small fluid filled spaces eventually amalgamating to form one large cavity. The follicular fluid is a mix of water, electrolytes, serum proteins and high concentrations of the steroid hormones secreted by the granulosa cells (Hirshfield, 1991). On the formation of the antral cavity the granulosa cells subdivide into two different populations (Latham et al., 1999; 2004), as previously discussed.

The pre-ovulatory surge of LH is the major influence on the ovulation of the dominant oocytes marking the end of the developmental process of folliculogenesis (Lee et al., 1996). In these final stages of follicle maturation after the pre-ovulatory LH surge, cumulus expansion (also known as mucification) occurs (Chen et al., 1990; Espey and Richards, 2002; Richards et al., 2002, Richards, 2005). The indirect actions of gonadotrophins stimulate the cumulus cells to produce hyaluronic acid which is a non-sulphated glycosaminoglycan, this then binds to the cumulus cells and expands the spaces between the cells, embedding them in a mucinous matrix (Chen et al., 1990; Salustri et al., 1990; Camaioni et al., 1993; Tirone et al., 1997). It is fundamentally important that the appropriate composition and assembly of the cumulus matrix occurs for successful ovulation, to aid the efficient passage of the oocyte through the oviduct, and for fertilization (Richards, 2005; Russell and Salustri, 2006)

Before ovulation the pre-ovulatory follicle must be exposed, and respond to, adequate LH and FSH levels in order for the eventual CL to secrete elevated amounts of progesterone to maintain normal function. As ovulation approaches, in addition to cumulus expansion the oocyte undergoes germinal vesicle (GV) breakdown with a formation of a polar body with subsequent oocyte arrest in metaphase II. The basement membrane surrounding the follicle ruptures at the onset of ovulation releasing the oocyte for fertilization (Hirshfield, 1991) and only after sperm penetration does final maturation and completion of meiosis occur.

Following ovulation luteinisation occurs, which is the process by which the follicular tissue structurally remodels with thecal and granulosa cell differentiating to form a functional CL, a process which can surprisingly occur in only a few hours (Richards et al., 1998; Richards et al., 2002). The formation of the CL is complemented by substantial changes in the vascularisation and the steroidogenic capacity of the follicle site. The development of capillaries from pre-existing blood vessels is recognised as an essential component for CL formation and function (Reynolds et al., 2000; Fraser et al., 2000). Furthermore, the expression of steroidogenic enzymes and steroids produced changes dramatically after luteinisation. The CL becomes a substitute site for progesterone production in addition to the luteal cells continually synthesising androstenedione and oestradiol. In addition prolactin plays a critical role in the CL maintenance in rodents throughout pregnancy but not in other mammals (Albarracin and Gibori, 1991). The regression of this endocrine gland occurs in two phases in the rodent, the first accompanied by a decrease in progesterone secretion known as functional regression, and the second occurring after the initial decline in progesterone, known as structural regression characterised by a decrease in size and weight of the CL (Stocco et al., 2007). Furthermore this regression of the CL occurs as a result of luteal cell death and replacement of the vascular supply (Goyeneche et al., 2003).

1.3. Control and regulation of follicle progression

The processes of folliculogenesis discussed above are under tight regulatory control, and communication between factors, both pituitary gonadotrophins and steroids, are well accepted. Furthermore, new evidence in recent years supporting the importance of intra-ovarian factors as part of this remarkable regulation has emerged despite limited knowledge of signalling and control. Together these factors are responsible for all aspects of control and regulation of follicle progression.

1.3.1. Gonadotrophins

The mammalian ovarian cycle is under the primary control of the hypothalamic gonadotropin-releasing hormone (GnRH) regulating the pituitary gonadotrophins,

FSH and LH, which form part of the essential hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.5). FSH and LH are both heterodimeric globular glycoprotiens which share a common α subunit with the β subunit determining the individual unique function (Gharib et al., 1990). FSH and LH are synthesised in the gonadotroph cells of the anterior pituitary with both hormones functionally active through their own receptors. Because activation of the HPG axis is crucial for the onset and progression of puberty and reproductive function, mutations affecting gonadotrophin actions have a major influence on subsequent developmental processes. In female mice that fail to secrete gonadotrophins as a result of a deletion in the GnRH gene (Cattanach et al., 1977) or after gonadotroph ablation (Kendall et al., 1991) or after disruption of the gene encoding the common α subunit (Kendall et al., 1995) have ovaries containing only primordial, primary and pre-antral follicles. These results indicated that gonadotrophins, although not required for follicle recruitment and initial development, are essential for development beyond the antral stage. Furthermore the FSHβ transgenic mouse show a similar phenotype of follicle development as described above, further indicating that FSH is the critical gonadotrophin required after this stage of development (Dierich et al., 1998; Abel et al., 2000). Collectively these studies highlight gonadotrophins to be key regulators in the maintenance of follicle growth at the onset of antral formation in the adult mouse.

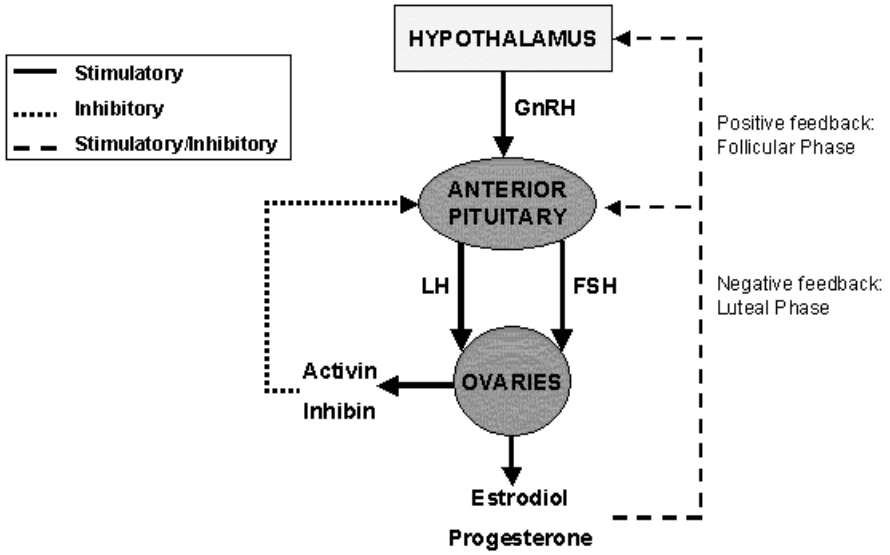

Figure 1.4: Female HPG axis, regulation of ovarian control by GnRH from the hypothalamus acting on the pituitary releasing FSH and LH. FSH and LH are secreted into the general circulation where they attach to receptors on the ovary, triggering follicle growth and subsequently ovulation. The ovary is stimulated to produce oestrodiol and progesterone which act in a positive stimulatory/or inhibitory affect depending on stage of oestrous cycle. Activin and inhibin are also produced locally and act on the anterior pituitary in an inhibitory manner.

Despite this well characterised role of the gonadotrophins in the development of early antral follicles, experiments using cultured large rat preovulatory follicles have demonstrated that LH and FSH have been additionally implicated in the increase of the apoptotic factors caspase 3 and 7 activities (Yacobi et al., 2004; 2007). High concentration of FSH and LH resulted in an increase in theca cell apoptosis but did not induce apoptosis in the granulosa cells in these follicles in culture (Yacobi et al., 2004). Although contradictory to the positive effects of FSH and LH, it has been suggested that these findings support previous concepts that thecal apoptosis maybe the physiologic process underlying gonadotrophin-induced ovulation (Murdoch and Gottsch, 2003).

1.3.1.1.FSH and FSH receptor expression and function

As mentioned FSH is well characterised in its roles associated with follicle growth and is considered essential to follicle survival, with receptor expression being reduced during atresia (Tilly et al., 1992). Furthermore FSH sensitivity plays an

important role in selection and progression of the antral follicle dominance (Uilenbroek et al., 1980; Tilly et al., 1992).

The FSH hypothesis dates back to 1978 (Brown, 1978) and is still believed to be the fundamental underlying mechanism for FSH action. The hypothesis suggests that individual follicles from within the initial cohort of recruited developing follicles have a FSH threshold level. Beyond this threshold the follicles must be stimulated by FSH to start their pre-ovulatory development and failure of FSH to be maintained above the threshold levels results in follicle atresia. It is thought that the FSH threshold is mediated by paracrine and autocrine mechanisms, and although discussed later in detail, androgens and activins have been identified as active participants (Hillier, 1991a, 1991b). The follicle(s) whose granulosa cells are most responsive to FSH (lowest threshold) become the first in the developing cohort to produce and secrete oestrogen, which then feeds back through the hypothalamic-pituitary axis to suppress FSH secretion (Figure 1.4). This decrease in pituitary FSH results in a reduction of circulating FSH which is insufficient to support the development of the remaining follicles (Johnson and Everitt, 2007). The remaining follicles most probably have elevated FSH threshold potentials, consequently they are unable to establish growth under the reduced FSH concentrations and become atretic (Hillier, 1994).

FSH requires the expression of its cognate receptor (R) to have a functional effect and in the rat FSHR mRNA has been localised to the granulosa cells of all sizes (Nakamura et al., 1991; LaPolt et al., 1992). Studies have demonstrated that on initiation of granulosa cell growth by stimulation with pregnant mare serum gonadotrophin (PMSG) to induce follicular development, FSHR mRNA expression and FSH binding sites increase. This initial increase in FSHR mRNA was subsequently demonstrated to decline after initiation of ovulation with hCG (LaPolt et al., 1992). Furthermore, similar observations have been demonstrated *in vivo* whereby the induction of ovulation by recombinant FSH suppressed FSH binding and FSHR mRNA expression, thus suggesting that the regulation of FSHR expression within the ovary may be biphasic by FSH itself. Thus, low doses of FSH

increase the number of FSH binding sites parallel to the increase of FSHR mRNA levels. In contrast, comparatively high doses of FSH down-regulate FSH receptor-binding sites and mRNA levels suggesting that there is a suppression of FSH gene expression and protein synthesis related to an increase in FSHR occupancy and internalization of FSH to the granulosa cells (LaPolt et al., 1992; Hsueh and LaPolt, 1992). Furthermore, studies using the HPG knockout mouse models have demonstrated that despite the absence of functional FSH ligand, FSHR mRNA is present suggesting that there may be additional factors that act to induce the expression of the FSHR (Adashi, 1994; Abel et al., 2003).

1.3.1.2.LH and LH receptor expression and function

The physiological functions of LH include direct stimulation of androgen production by the theca cells (Zeleznik and Hillier, 1984). Furthermore, LH is required to maintain the dominant status of the pre-ovulatory follicles. However there is evidence, as with FSH, that excessive exposure of LH to the ovaries can have adverse effects on the development of the selected pre-ovulatory follicles (Chappel and Howles, 1991; Hillier, 1993). Premature luteinisation of the granulosa cells can occur in addition to having detrimentally effecting oocytes development (Chappel and Howles, 1991; Picton and McNeilly, 1991). Therefore it has been suggested that whereby there is a threshold for FSH stimulation there may be a ceiling effect of LH action (Hillier, 1993). When these ceiling levels are exceeded during the mid-cycle surge of LH, further division of the granulosa cells ceases and the process of luteinisation occurs. This ceiling effect of LH has been further explored *in vitro* by monitoring granulosa cell proliferation and steroid production. On treatment with low dose of LH, steroidogenesis of the granulosa cells was enhanced, but on treatment with a high dose of LH, enhanced synthesis of progesterone was observed accompanied by a suppression of aromatase activity and an inhibition of growth (Overes et al., 1992; Yong et al., 1992).

As mentioned LH exerts its effects through binding to its cognate receptor, which belongs to the seven transmembrane domain G-protein associated receptors (Segaloff and Ascoli, 1993). Within the ovary, LHR can be detected in theca cells where they

regulate androgen production, as well as in luteinising granulosa and luteal cells where they regulate progesterone production. LHRs are also located on the granulosa cells of pre-ovulatory follicles which have been adequately stimulated by FSH where they are coupled to aromatase (Richards et al., 1987).

1.3.2. Steroid control

Sex steroids play a define role in the growth and differentiation of all reproductive tissue with oestradiol and progesterone being recognised as the major players in the mammalian female. Steroid secretion by the antral follicle involves the interplay of androgens, oestrogens, and progestins with their impact on ovarian function determined by the availability of the ligand, receptor expression and the repression or induction of relevant regulatory genes (Drummond et al., 2002). Within the follicle a two-cell two-gonadotrophin model exists which describes the interaction between the theca and granulosa cells in addition to the stimulatory effects of the gonadotrophins, all of which are necessary for steroid synthesis and oestrogen production (Figure 1.5) (Drummond, 2006).

Both the granulosa and thecal cell compartments contribute to follicular fluid and serum levels of steroids with the granulosa cells being the major cellular source of the production of the ovarian hormones, oestradiol and progesterone, whereas the theca cells are the site of androgen production (Figure 1.5) (Drummond, 2006). Much of the established data on the role and regulation of ovarian steroid comes from experiments investigating nuclear receptor knockout mouse models which consequently lead to the disruption of the steroid signalling mechanisms, hence preventing steroids fulfilling their unique roles in follicle development. An overview of the production and functions of progestins, androgens and oestrogens within the ovary are now discussed.

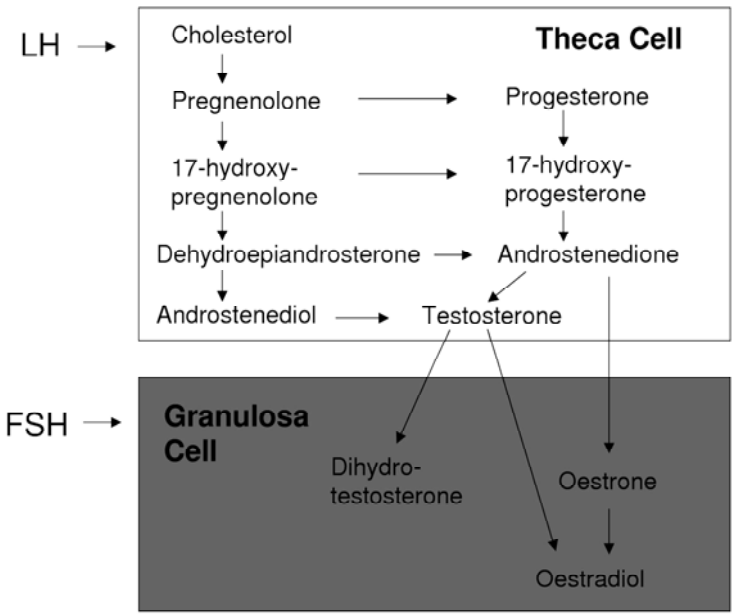

Figure 1.5: Steroid biosynthesis within the granulosa and thecal cells of the ovary. Initially within the theca cholesterol is converted to pregnenolone which in turn is metabolised to testosterone through a series of substrates. This two cell two gonadotrophin model is initiated when androgens produced by the theca are transported to the granulosa where they are aromatised into oestrogen (Drummond, 2006).

1.3.2.1.Progesterone production and function

Progesterone is synthesised predominantly in the theca cells, stromal cells and luteal cells (Duleba et al., 1999) and is most notably associated with playing important functional roles in ovulation, implantation and the maintenance of a viable pregnancy (Graham and Clarke, 1997). As with most steroids progesterone actively functions on binding to its own receptor(s). Two forms of the progesterone receptor (PR) have been identified namely PR-A and PR-B, with each having a different functional role (Spelsberg et al., 1972; Kastner et al., 1990; Conneely et al., 2002; Conneely et al., 2003). It has been demonstrated that PR-A is dominant over PR-B and that the ratio of these two receptors can determine cellular response. Hence different groups of genes are believed to be controlled by the different receptors in response to progesterone binding (Tora et al., 1988; Meyer et al., 1992; Tung et al., 1993; Vegeto et al., 1993). PRs are localised to the thecal layer of cells surrounding small antral follicle and have been identified on the granulosa of pre-ovulatory follicles which

had been exposed to LH/hCG inducing the LH surge or were undergoing luteinisation (Park and Mayo, 1991) thus supporting a role for progesterone and expression of its receptors in ovulation and luteinisation.

The use of transgenic mouse models has provided a wealth of information into the mechanisms surrounding PR function (Lydon et al., 1995), with the PR KO females presenting with functionally normal ovaries. However, these animals fail to ovulate even after gonadotrophin stimulation with their granulosa cells undergoing luteinisation which interestingly suggests that PR is not essential for CL formation (Robker et al., 2000). These finding are somewhat controversial as it has been previously acclaimed that progesterone acting as least partly via the PRs are required for luteinisation (Natraj and Richards, 1993). It is now acknowledged that on the development of PR-A and PR-B individual KOs, PR-A is the necessary PR for ovulation with these females being infertile but not PR-B as these KO mice are functionally fertile (Mulac-Jericevic et al., 2000; Mulac-Jericevic et al., 2003). This further supports the evidence that these two PRs mediate alternative actions of progesterone (Mulac-Jericevic and Conneely, 2004).

There is additional evidence that some actions of progesterone, including inhibition of apoptosis and insulin-dependent mitosis, are mediated by an alternative non-genomic mechanism of PR (Peluso et al., 2006). These alternative mechanisms are probably mediated by recently discovered progesterone binding proteins or progestin membrane receptors although not extensively studied (Peluso and Pappalardo, 1998; Bramley, 2003; Peluso, 2003; Cai and Stocco, 2005; Peluso, 2006).

1.3.2.2.Androgen production and function

One of the most recognisable and important functions of androgens in the mammalian ovary as previously stated is their role in the synthesis of oestrogen, serving mainly as a substrate of P450 aromatase mediating the conversion to oestrogen (Dorrington et al., 1975; Tetsuka and Hillier, 1996; Hillier and Tetsuka, 1997; Drummond, 2006). This increase in oestradiol production in conjunction with an increase in progesterone production facilitates the roles of androgens in

supporting follicle growth by enhancing the FSH mediated differentiation of granulosa cells (Weil, 1999; Drummond, 2006). Furthermore androgens are associated with female reproductive pathologies, most notably polycystic ovarian syndrome (PCOS) in humans, a condition resulting from an excess production of ovarian androgen (Ehrmann, 2005).

Exogenous androgens have been shown to exert both inhibitory and stimulatory effects at different stages of follicular development (Walters et al., 2008). Studies analysing the functions of androgens have demonstrated that testosterone, androstenedione and dihydrotestosterone can all stimulate follicle growth (Murray et al., 1998; Wang et al 2001). However androgen function has also been implicated in the regulation of follicle atresia by the indirect inhibition of FSH-stimulation in a developmental manner (Harlow et al., 1988) and directly through induction of follicle atresia (Hillier et al., 1979; Azzolin et al., 1983). However as a result of androgens capabilities of steroid conversion with the potential to exert further indirect actions, results from many studies are difficult to interpret accurately and present conflicting inhibitory and stimulatory data (Walters et al., 2008).

The mouse androgen receptors (AR) are mainly located on the granulosa cells and in cells within the ovarian stroma (Schreiber and Ross 1976; Hirai et al., 1994; Tetsuka et al., 1995) and have been recently localised to the mouse oocytes (Szoltys and Slomczynska 2000; Gill et al., 2004). Depending on species investigated expression of AR occurs in the granulosa cells of mainly preantral and antral follicles, with evidence that immunoexpression is more intense for AR within the cumulus and antral granulosa cells, with reduced expression in the peripheral layers of the granulosa in the rat (Tetsuka et al., 1995). The expression of AR declines and androgens are metabolised as opposed to exerting direct effects on folliculogenesis during the stages of late preovulatory development (Hillier and Tetsuka, 1997). More recently AR KO mice have been produced efficiently utilising the Cre/LoxP system providing definite proof for AR mediated actions within the ovary and in follicle development (Yeh et al., 2002; Hu et al., 2004; Shiina et al., 2006; Walters et al., 2007). These studies have emphasised the importance of AR in age related

female fertility, through optimising conditions for follicle growth and play vital roles at ovulation (Yeh et al., 2002; Hu et al., 2004; Shiina et al., 2006; Walters et al., 2007; Walters et al., 2008).

1.3.2.3.Oestrogen production and function

The local intra-follicular actions of oestrogen have been historically recorded with its actions vital to female reproductive physiology preparing the female reproductive tract for conception (Hisaw, 1947). The biosynthesis of oestrogen is first detected in the late pre-antral follicles where it requires the cooperation of both the granulosa and theca cells and FSH and LH, as described above (Figure 1.6). Small antral follicles are incapable of producing androgen substrate for aromatisation to oestrogen (Carson et al., 1981). However, subsequent follicle growth is characterised by an increase in aromatase activity, androgen synthesis and hence oestrogen production (McNatty, 1982; Drummond, 2006). This increase of oestrogen is also responsible for facilitating granulosa cell differentiation including the induction of receptor systems for FSH, LH and prolactin influencing post-receptor mechanisms (Richards, 1980; Drummond, 2006). Oestrogen plays a major role in the endocrine control of follicular development whereby it acts to suppress the FSH release by the pituitary gland (Figure 1.4). Furthermore, this suppression subsequently minimises the stimulation of additional pre-ovulatory follicles and additionally causes discharge of the mid-cycle surge of LH by the pituitary gland triggering ovulation (Hillier, 1990, 1994).

Oestrogens, as with the androgens and the progestins, signal via its receptors, ERα and ERβ (Green et al., 1986; Kuiper et al., 1996; Chu and Fuller 1997; Petersen et al., 1998). ERβ is predominantly expressed within the ovary increasing in expression coinciding with granulosa cell proliferation, with ERα mRNA levels remaining constant (Byers et al., 1997; Drummond et al., 1999). Utilising the genetic manipulation of KO models the absence of these two receptors individually ERα (ERKO) and ERβ (BERKO) or collectively (αβERKO) has been investigated. Although these models are not oestrogen free, a further transgenic model the oestrogen depleted mouse which lacks functional aromatase (ArKO) has been further

exploited to investigate the essential role of oestrogen on ovarian development (Lubahn et al., 1993; Couse et al., 1997; Krege et al., 1998; Couse et al., 1999; Dupont et al., 2000; Britt et al., 2001).

The main findings from these comprehensive studies conclude that ERKO females are phenotypically acyclic, infertile and possess hyperemic ovaries devoid of CLs. Furthermore, folliculogenesis is arrested at the antral stage with follicles becoming cystic and haemorrhagic (Lubahn et al., 1993; Couse et al., 1997). However, on treatment with gonadotrophins prepubertal ERKO mice can ovulate (Rosenfeld et al., 2000) suggesting that the processes of folliculogenesis, ovulation and CL formation can occur in the absence of ERα, although not efficiently. Furthermore, prolonged exposure of ERKO mice to a GnRH antagonist prevent the haemorrhagic cyst formation (Couse et al., 1999 a, b) indicating that their ovarian phenotype manifests as a consequence of elevated LH levels (Couse et al., 1999b; Schomberg et al, 1999). Interestingly, the BERKOs have small ovaries presenting with some arrested follicular development but these individuals are fertile with normal gonadotrophin levels. Although fertile, this process is severely compromised with a reduced number of offspring which is consistent with the reduced numbers of CL present and hence reduced ovulation (Krege et al., 1998). The ovarian phenotype of the αβERKO is again distinct from that of the ERKO and BERKO (Couse et al., 1999b; Dupont et al., 2000). In the absence of both receptors ovaries undergo follicular trans/re-differentiation to phenotypically resemble seminiferous cord like structures containing Sertoli like cells as a result of elevated LH. It can therefore be concluded from these studies using the ER knockout models that both ERα and ERβ have significant roles in the maintenance of fertility and ovarian function.

Furthermore, the ArKO mouse oestrogen depletion model defines how far follicles can grow in the total absence of oestrogen (Fisher et al., 1998; Britt et al., 2001). The morphology of these ovaries consists of abnormal follicles which are represented by seminiferous tubule-like structures with Sertoli like cells apparently arising from the trans/re-differentiation of granulosa cells (Britt et al., 2001) similar to that observed in the αβERKO models (Couse et al., 1999 a b; Dupont et al., 2000). In

addition the ArKO females present with retarded follicle development as follicles arrest at the antral stage and, combined with no ovulation these finding emphasise the importance of oestrogen is follicle development. Overall it can be notably concluded from these KO studies that the roles of oestrogen and its functional receptors are obligatory for normal progression of folliculogenesis.

1.3.3. Intra-ovarian regulators

It has been clearly acknowledged that gonadotophins, FSH and LH (Section 1.3.1) and steroid hormones, progestins, androgens and oestrogens (Section 1.3.2) are fundamental in the control, regulation and function of ovarian development and maintenance of the cyclic processes of folliculogenesis. In addition to these local and systemic endocrine factors, intra-ovarian regulators have been identified to be critical in supporting these intricate processes surrounding folliculogenesis. Intra-ovarian factors synthesised within the follicle maintain direct intra-follicle control of differentiation, granulosa cell proliferation and apoptosis accompanying and moderating the gonadotrophic control of these processes. Understanding this intra-ovarian communication between the oocytes and granulosa cells and the importance of the bi-directional control is vital in complementing what is known regarding the endocrine control of the ovary. A wealth of emerging evidence in the past 10 years supporting the actions of these autocrine/paracrine locally produced factors has arisen and despite the collective data of numerous *in vitro* studies surrounding these factors, the coordination and control of these mechanisms *in vivo* are not clearly defined. In the next section, the role of a number of intra-ovarian factors are discussed in particular members of the TGFβ family, activin, inhibin, follistatin, BMPs and GDF9.

1.3.3.1.TGFβ family signalling

The TGFβ superfamily consists of over 30 extracellular signalling molecules which are structurally related but functionally diverse proteins. These TGFβ molecules collectively regulate many functions in a variety of tissues, and a number have been demonstrated to be locally expressed within the ovarian follicle; GDF9, BMP15,

inhibins, activins, TGFβ, BMPs 2, 4, 6, 7 and AMH (Findlay, 1993; Nishimori and Matzuk, 1996; Knight and Glister, 2001; Richard, 2001; McNatty et al., 2001).

The signalling of TGFβ molecules is brought about by transmembrane serine/threonine kinase receptors (R) type I and type II receptors, both of which are essential for the transduction of TGFβ members into the cell. Collectively five types of RII, which are considered constitutively active and seven types of RI have been identified, providing a variety of potential signalling requisites and functional outcomes (Knight and Glister, 2003). The activation and translocation of TGFβ molecules is assisted by the involvement of Smad proteins. The Smad proteins are ubiquitously expressed in nearly all cell types in the body and to date 8 different Smad proteins have been identified which can be classified based on their active function. They are either receptor activated Smads 1, 2, 3, 5 and 8, common-partner Smad 4 or inhibitory Smads 6, 7. On binding of the TGFβ ligands to the transmembrane serine/threonine kinase RII receptors, activation and dimerisation with RI occurs forming a tetrameric signalling complex. Depending on the combination of receptors that are activated different activator Smads are recruited through phosphorylation by the RI receptor. The receptors activate Smads and subsequently form oligomeric complexes with the common Smad 4 after which translocation to the nucleus occurs. On translocation target genes are then regulated via interaction with a multitude of transcription factors, co-activators and co-repressors (Kaivo-oja et al., 2006). In general terms the Smad 2/3 pathways is utilised by TGF-βs, activins and GDF9 whereas the Smad 1/5/8 pathway is utilised by BMP15, BMP2, 4, 6 and 7 (Table 1.1). More than one pathway can be transduced by the same ligand and in addition to the implications of extracellular, intracellular and transcriptional regulation and accessory proteins, combinations of complexes and regulatory outcomes are multiple (Findlay et al., 2002; Kaivo-oja et al., 2006). The inhibitory Smads do as they suggest and can block TGFβ signalling by either the binding of the inhibitory Smad 7 to the RI or by Smad 6 competing with activated Smad 1 for binding to the Smad 4. Furthermore, Smad 7 can inhibit the activation of both the TGFβ/activin and BMP pathways, whereas Smad 6 only inhibits BMP signalling (Attisano and Wrana, 2002; Shi and Massague, 2003).

Although Smad proteins are ubiquitously expressed in most cell types and TGFβ family members are involved in signalling elsewhere, selective disruption of the individual Smad genes has highlighted some important issues regarding their function with evidence of specific action of Smad 3 in the ovary. Although not ovarian specific it is important to recognise the following Smad KO phenotypes, emphasising their diverse roles. Mice homozygous and heterozygous for KO of Smads 1, and 5 result in embryonic death suggesting roles for these proteins in the regulation of embryogenesis (Arnold et al., 2006). To date there is unpublished evidence that the Smad 8 mutants are viable and fertile (Z.Huang et al., in Pangas et al., 2008). Partial loss of exon 1 of Smad 7 results in smaller mice with altered B cell responses as a result of a defective immune system (Li et al., 2006). Smad 6 mutant homozygous mice display with cardiovascular impairments (Galvin et al., 2000). Although not mentioned in these publications it cannot be ruled out that these Smads are involved in ovarian signalling with the published phenotypes being of interest.

Table 1.1: Summary of identified signalling pathways of TGFβ superfamily members within ovary (adapted from Kaivo-oja et al., 2006).

Ligand	Expressed by	RII	RI	Smads	References
GDF9	Oocytes	BMPRII	ALK5	Smad 2/3	Mazerbourg et al., 2004, Roh et al., 2003; Vitt et al., 2002
BMP15	Oocytes	BMPRII	ALK6	Smad 1/5/8	Moore et al., 2003
BMP6	Oocytes, granulosa	BMPRII/ActRIIA/B	ALK2/3/6	Smad 1/5/8	De Caestecker, 2004; Shimasaki et al., 2004; Ebisawa et al.., 1999
TGFβ 1, 2, 3	Granulosa, theca	TβRII	ALK5	Smad 2/3	Lin et al., 1992; Franzen et al., 1993
Activin A/B	Granulosa	ActRIIB	ALK4	Smad 2/3	Attisano et al., 1992; ten Dijke et al., 1994a
Inhibin α	Granulosa	ActRIIA/B	?	?	Mathews et al., 1991
BMP-2	Granulosa	BMPRII/ ActRIIA	ALK3/6	Smad 1/5/8	Rosenzweig et al., 1995, Yamaji et al., 1994
BMP-3	Granulosa	ActRIIB	?	?	Gamer et al., 2005
AMH	Granulosa	AMHRII	ALK2/3/6	Smad 1/5/8	Baarends et al., 1994, Visser et al., 2001, Gouedard et al., 2000, Jamin et al., 2002
BMP4	Theca	BMPRII/ ActRIIA	ALK3/6	Smad 1/5/8	Rosenzweig et al., 1995, Yamaji et al., 1994, ten Dijke et al., 1994a
BMP7	Theca	BMPRII/ ActRIIA	ALK2/3/6	Smad 1/5/8	ten Dijke et al., 1994b, Yamashita et al., 1995

The Smad 3 homozygous null mice are viable although fertility is somewhat reduced compared with wild type mice (Tomic et al., 2002; 2004). In the absence of Smad 3 the ovarian surface epithelium appears distinctly morphologically altered, being thickened with enlarged cuboidal cells instead of being flat with thin cells as observed in the wildtype. Furthermore it has been suggested that Smad 3 may regulate growth of primordial follicles to the antral stage although it may not affect the size of the primordial follicle pool at birth (Tomic et al., 2002). In addition it has been suggested that Smad 3 may regulate the expression of Bax and Bcl-2, but not Bcl-x, Cdk-2, and PCNA suggesting a role in programmed cell death and not proliferation. Collectively, these data suggest that Smad 3 specifically plays an important role in the regulation of ovarian follicle growth and female fertility (Tomic et al., 2002; Symonds and Tomic, 2003).

1.3.3.2. Activin, inhibin and follistatin

The inhibins and activins have a basic molecular structure, made up of three separate subunits which form the various heterodimers (Figure 1.6).

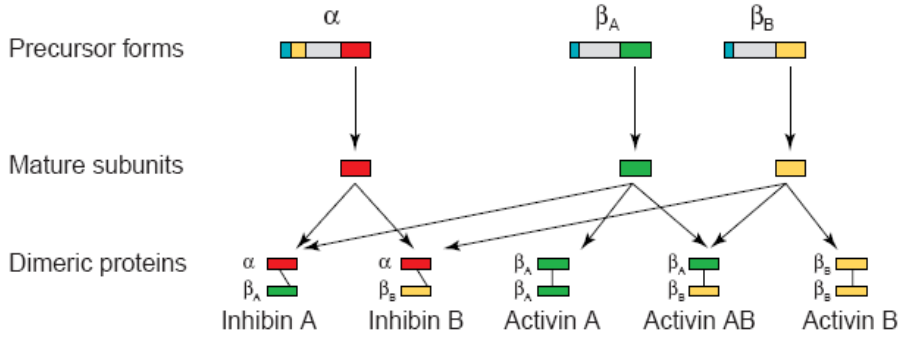

Figure 1.6: Basic molecular structure inhibins and activins. Subunits are initially synthesised as precursors. The α subunit can associate with either βA or Bβ to form inhibin A or inhibin B. The β subunits can dimerise to form the activins (adapted from Ethier and Findlay, 2001).

A wealth of information surrounding the actions of activin, inhibin and follistatin has been documented since their discovery and despite the overwhelming evidence of their action there are still many unanswered questions surrounding their function.

Much work to data has focused on the use of *in vitro* studies, using isolated follicles, granulosa cell, theca cell and oocyte culture systems. More recently the use of transgenic mouse models have been exploited to support the critical roles of activins, inhibins and follistatin providing evidence regarding their roles in ovarian development and functional regulation (Knight and Glister, 2001; Pangas and Matzuk, 2004). Compelling evidence has been collated to implicate the involvement of these intra-follicular mediators as autocrine/paracrine modulators with effects on the granulosa cells, theca cells, the oocytes and actions in the CL (Knight and Glister, 2003) (Figure 1.7). The general function of these three TGFβ molecules, activin, inhibin and follistatin, in follicular development are reviewed in the following sections

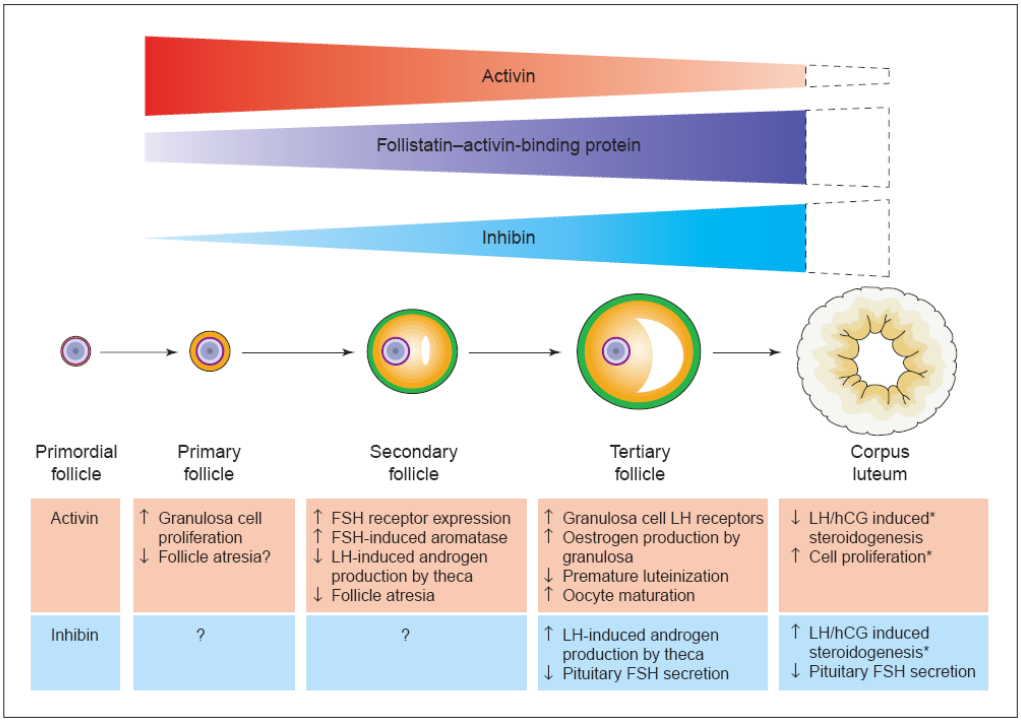

Figure 1.7: Summary diagram of inhibin, activin and follistatin expression and functions in granulosa cells at different stages of follicle development, indicating their putative regulatory functions at the intraovarian and peripheral level. ↑↓ indicate increase or decrease of function. Follistatin can neutralise the biological activity of activin through its capacity to bind activin. (Note that expression of inhibin A and follistatin is maintained in primate corpus luteum, but declines after ovulation in other species. * indicate roles restricted to primate species) (Knight and Glister, 2001).

1.3.3.3. Activin

Activins have been shown to play a host of roles in the developing ovary in a number of different species, with its most notable roles being its involvement in granulosa cell proliferation and in FSH regulation (Figure 1.7) (Findlay, 1993; Hillier, 1994; Mather et al., 1997). In the presence of FSH activin responds biphasically, acting in an inhibitory fashion at low doses and stimulatory at high doses (Xiao et al., 1992). It has been suggested that follicle progression from the late preantral (gonadotrophin independent) to the early antral stage (gonadotrophin dependent) may be mediated by the actions of activin promoting the expression of the FSH receptor on the granulosa cells (Hasegawa et al., 1988; Xiao et al., 1992). This has been further supported during studies demonstrating that after treatment with activin A growth of isolated preantral follicles from immature rats but not same stage follicles from adult rats was induced, demonstrating the role of activin in the acquisition of granulosa cell responsiveness to FSH by regulating the FSH receptor in these immature follicles (Findlay, 1993; Findlay and Drummond, 1999). It has also been shown that cultured granulosa cells harvested from both small and large follicles exhibit enhancing proliferation after treatment with activin, supporting the intrafollicular autocrine/paracrine action of activin on granulosa cell proliferation and differentiation (Li et al., 1995; Miro and Hillier, 1996; Zhao et al., 2001). Furthermore, an overproduction of activin as identified in the mouse model with a targeted deletion of the inhibin α subunit was shown to be associated with uncontrolled proliferation of the granulosa cells with the subsequent development of ovarian tumours, thus further supporting activins role in granulosa cell proliferation (Matzuk and Bradley, 1992).

Activin receptors are expressed in the oocytes (Cameron et al., 1994; Sidis et al., 1998) and evidence from studies performed in the monkey and rat suggests that activins derived from the cumulus granulosa cells affect nuclear and cytoplasmic maturation of oocytes (Sadatsuki et al., 1993; Alak et al., 1996). On deletion of the activin type IIB receptor follicle development is arrested at the early antral stage, consistent with activin being functionally active in granulosa cell proliferation and differentiation (Nishimori and Matzuk, 1996). However, this coincided with a

reduction in plasma FSH, evidently compromising the assessment between the indirect effects of reduced pituitary FSH and the direct intra-ovarian effect of activin receptor IIB deletion (Nishimori and Matzuk, 1996).

Activin activity is neutralised by follistatin and since one of the primary actions of activin is to induce FSHR expression in the developing follicle, the efficiency of this process is best achieved in the absence of follistatin. *In vitro* studies have demonstrated that granulosa cells from small follicles have reduced follistatin compared to larger follicles, which are more likely to acquire FSH receptors. This supports the action of activins in promoting FSHR acquisition and gonadotrophin follicular dependence (Shimasaki et al., 1989; Nakatani et al., 1991).

1.3.3.4. Inhibin

Two forms of inhibin exist (A and B) both of which are present in the mouse ovary (Knight, 1996). Inhibin plays a direct role in the negative feedback system (Figure 1.3) modulating pituitary FSH secretion during follicle growth. Dominant follicles produce increasing amounts of inhibin and follistatin which in turn reduces the activity of activin up-regulation of thecal androgen production, the increase in inhibin reduces pituitary FSH secretion and acts to maintaining follicle dominance. There have been inconclusive results surrounding the identification of inhibin specific receptors and it is thought that they mediate most of their function through competitively binging to the activin type II receptors although this does not then stimulate type I receptor phosphorylation (Zimmerman and Mathews, 1996; Martens et al., 1997; Chapman and Woodruff, 2001). Furthermore, TGFβ type III receptor, also referred to as betaglycan has been shown to promote a high affinity associated between inhibin and activin type II receptor, acting as a putative inhibin co-receptor, thereby providing a mechanism of activin antagonism even at low concentrations of inhibin (Lewis et al., 2000; Bernard et al., 2001; Gray et al., 2002).

Studies in cattle have demonstrated that inhibin B concentrations from follicular fluid are inversely correlated to follicle size, whereas inhibin A concentrations are positively correlated with follicle size suggesting a developmental transition in their

individual functions (Knight and Glister, 2003). Additionally studies using a number of different species including the rodent (Hsueh et al., 1987) have demonstrated that in thecal cells inhibin A, the product of gonadotrophin-responsive osetrogen-active granulosa cells, can enhance LH induced androgen production. These findings have been further supported using cultures of intact rat follicles. A decrease in androgen secretion was shown after incubation with a neutralising antibody against inhibin, supporting the positive action of endogenous inhibin on thecal androgen production (Smyth et al., 1993).

1.3.3.5. Follistatin

Follistatin (FST) is structurally unrelated to the inhibins despite having similar function in suppressing pituitary FSH (Ueno et al., 1987). The main physiological function of FST is its ability to neutralise the actions of activins and, to a lesser extent inhibins through binding to the β subunit (Shimasaki et al., 1991). In addition the binding affinity of FST for activin is similar to that of activin binding to its own receptors (Mathews, 1994; Knight and Glister, 2003). Furthermore, FST has been shown to reduce oocyte developmental competence and neutralise the effects of both endogenous and exogenous activin, consistent with its functional role as an activin-binding protein (Silva and Knight, 1998). As a result of its activin binding properties, FST has also been demonstrated to be involved in promoting follicle atresia, associated with a decrease in oestradiol and inhibin production and an increase in progesterone or luteinisation, depending on the stage of follicular growth (Knight and Glister, 2003). There are reports using bovine follicles which show that intrafollicular concentrations of FST do not vary throughout growth, hence suggesting a developmental regulated bio-availability of activin A, due to the effects free activin A/inhibin (Knight and Glister, 2003). However, expression of FST has been shown to be up-regulated in granulosa-lutein cells as a result of the administration of hCG, suggesting a possible role as an important regulator of the gonadotrophin-dependent luteal support mechanism (Tuuri et al., 1994). Although mostly derived from *in vitro* data these investigations into the actions of activins, inhibins and follistatin in the ovary clearly suggest that these are regulatory paracrine compounds are actively involved in folliculogenesis (Knight and Glister, 2001).

1.3.3.6.GDF9 and BMP15

Two oocyte expressed TGFβ family members which have been extensively studied in recent years are GDF9 and BMP15, both of which are essential for ovarian development and female fertility (Juengel et al., 2004).

Evidence suggests that oocyte specific GDF9 acts as a paracrine regulator involved in early follicle development, with its expression beginning in primary follicles in the mouse. Much of what is known regarding the function of GDF9 has come from studies using the transgenic GDF9 mouse KO model. There is now no doubt that GDF9 is fundamentally important in the development of the follicle. On deletion of GDF9, granulosa cell proliferation significantly decreases accompanied by abnormal oocyte growth with follicles failing to develop beyond the primary stage (Dong et al., 1996; Carabatsos et al., 1998). These abnormal follicles remain steroidogenically active and are arranged in clusters resembling corpora lutea, in addition, cells within these abnormal follicles have been shown to express luteal markers. Furthermore, it has been demonstrated that these follicles fail to undergo cell death suggesting that the granulosa cells of these follicles require GDF9 for continued growth but also to become competent to undergo apoptosis (Elvin et al., 1999). However, this failure to undergo cell death may be due to follicle arrest occurring at the primary stage of development where apoptosis is uncommonly detected. Furthermore, an up-regulation of kit ligand and inhibin α in the primary follicles suggests that these two secreted factors expressed by the granulosa cells may be directly regulated in a paracrine fashion by GDF9. This extensive transgenic mouse study into the molecular mechanisms surrounding the intra-ovarian regulation by GDF9 has contributed greatly to the understanding of this TGFβ family member. *In vitro* studies further support evidence that GDF9 is involved in the early stages of follicle development whereby oocyte derived GDF9 has been shown to stimulate the proliferation and suppress the FSH-induced differentiation of rat granulosa cells (Vitt et al., 2000).

BMP15 is also an oocyte-derived factor but, compared to GDF9, mice lacking functional BMP15 have a relatively normal folliculogenesis pattern with impairment observed at ovulation and the ability for the oocytes to be fertilised thus reducing overall fertility (Yan et al., 2001b; Wu and Matzuk, 2002). A more extreme phenotype is observed in naturally occurring point mutations of BMP15 in the sheep (Inverdale and Hanna) with the homozygous BMP15 mutants, FecX1/FecX, having their follicular development arrested at the primary stage, similar to the GDF9 mouse (Galloway et al., 2000; McNatty et al., 2001). Interestingly the heterozygous individuals have an increased ovulation rate resulting in multiple pregnancies suggesting a dose depended effect of BMP15 on folliculogenesis in the sheep (Galloway et al., 2000; Yan et al., 2001b). This dose dependent mechanism has also been identified in the DAZL KO mouse whereby heterozygous expression results in multiple pregnancies compared to the Wt homozygous (Section 1.6.1) (McNeilly et al., 2000). Furthermore, although not well documented the function of BMP15 has been shown to modulate FSH action in rat granulosa cells by suppressing FSHR expression (Otsuka et al., 2001).

In addition to these studies into single mutations of GDF9 and BMP15, heterozygous double crosses generating $GDF9^{+/-}$; $BMP15^{-/-}$ have been investigated. The generation of these mice show enhanced Bmp15 null phenotype (Yan et al., 2001b). Furthermore cumulus cells from these mutant mice were unable to adhere to the oocyte, and no cumulus expansion was observed, suggesting that each of these ligands use the same signalling machinery during the periovulatory period of folliculogenesis (Yan et al., 2001b).

Collectively these results support the roles of principally GDF9 in rodents and BMP15 in sheep in the early stages of follicle development associated with granulosa cell proliferation, and there is further evidence that GDF9 and BMP15 may play roles in the later stages of folliculogenesis possibly associated with FSH modulation and cumulus expansion (Elvin et al., 1999; 2000; Yan et al., 2001b; Juengel et al., 2002). Furthermore, there is recent evidence which suggests that heterodimers can

exist when these subunits are co-expressed *in vitro* but their biological activity remains to be tested *in vivo* (Liao et al., 2003).

1.3.3.7.Anti-Müllerian hormone

Anti-Müllerian hormone (AMH) is most notably recognised as having a role in inducing müllerian duct regression in the male fetus during development (Josso et al., 2001). However, AMH is a further member of the TGFβ superfamily which has been investigated and implicated in intra-ovarian signalling. AMH is first detected in the granulosa cells of the recruited primordial follicles, suggesting a possible role for AMH in controlling follicle growth (Durlinger et al., 1999; Durlinger et al., 2001). Interestingly, AMH is not expressed in follicles following dominance selection, indicating that the pattern of expression may have an important role in the regulation of both the number of growing follicles and their further selection for ovulation (Visser et al., 2007). Data from *in vitro* studies using rat pre-antral follicles have further demonstrated that AMH can enhance the FSH-induced increase in follicle diameter and cell number (McGee et al., 2001). Furthermore, the AMH null mouse model has been used to investigate the roles of AMH in folliculogenesis *in vivo* (Durlinger et al., 1999). AMH null mice display an increased recruitment of primordial follicles accompanied by reduced FSH levels. Interestingly, these mice do not have proportionally more preovulatory follicles suggesting that in the absence of AMH, follicles may be more sensitive to FSH (Durlinger et al., 1999; Visser et al., 2007). In comparison over expression of AMH leads to the inhibition of Müllerian duct differentiation in females, resulting in a blind vagina with no detectable uterus or oviducts. At birth the ovaries of these females have a reduced number of germ cells which are subsequently lost and somatic cells organise into structures resembling seminiferous tubules. Furthermore these structures are then thought to degenerate as they are undetectable in adult females (Behringer et al., 1990). Together these models of AMH depletion and over expression clearly indicate an imperative role for AMH in not only sexual differentiation but on follicle development and recruitment.

1.4. Programmed cell death

The emphasis of the first part of this review focused on the importance of engagement between control and regulation of factors supporting the development of primordial germ cells and follicles throughout folliculogenesis. These processes are without a doubt controlled by a substantial number of identifiable molecules, including the gonadotrophins, steroids and intra-ovarian growth factors which communicate and principally act as survival factors. Parallel to these complex interactions supporting development are the opposing events of programmed cell death. The interaction between the factors that promote survival and those that leads to atresia of follicles, oocytes and CLs are crucially important in ovarian biology.

The first description of apoptosis was within the rabbit ovary to which the process was call chromatolysis (Flemming, 1885). The ultrastructural features of apoptosis include condensation of the nuclear chromatin into circumscribed masses, convolutions of the nuclear and cellular outline, fragmentation and budding of the cell and the production of the membrane bound apoptotic bodies, and phagocytosis of the apoptotic bodies by macrophages. These descriptions are still recognised today as the main characteristics of apoptosis (Hussein, 2005).

Apoptosis can be generally described as a means for multicellular organisms to eliminate unwanted cells in response to developmental signals or toxic stimuli without the release of toxic cell contents, as occurs with necrosis (Quirk et al., 2003). Apoptosis can be achieved by two different response mechanisms: one mechanism is triggered by the binding of extrinsic death molecules to cell surface receptors (death receptor mediated events) while the other is generated by intrinsic mitochondrial mediated events arising from within the cell (Figure 1.8) (Danial and Korsmeyer, 2004; Hussein et al., 2005; Jin and El-Deiry 2005).

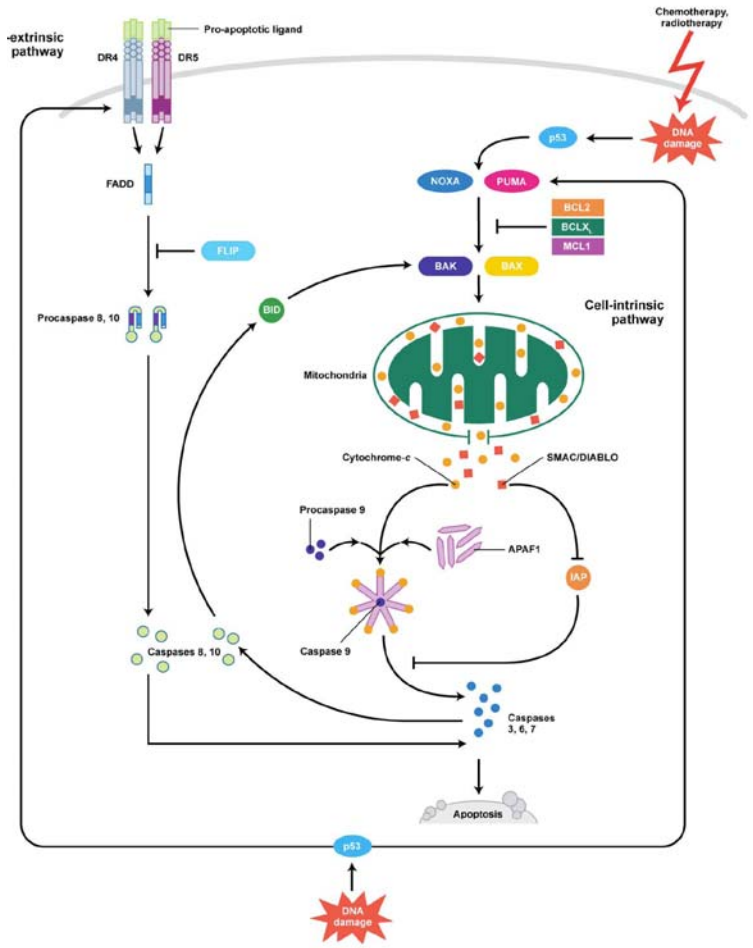

Figure 1.8: Intrinsic and extrinsic apoptosis signalling pathways, both of which are inducible in the granulosa cells (image: www.researchapoptosis.com/.../signaling/index.m).

The focus of this current section is mainly on the natural processes of apoptosis in the ovary and the factors involved. However it is important to note that pathological insults, in the form of either environmental biohazards or clinical therapies such as chemotherapeutic agents, can contribute to premature ovarian failure and subsequent infertility by interfering in the intrinsic program of apoptosis present in oocytes (Tilly, 2001).

1.4.1. Follicle atresia

Of the original 26,000 germ cells observed prenatally in the mouse approximately 10-15,000 of this germ cell reserve die prior to birth (Dean, 2002). For the majority germ cells which survive this initial wave of apoptosis in the fetal and newborn

ovary, their fate within follicles is not any better as apoptosis continues to decimate the oocyte pool through a process generally referred to as follicle atresia (Kim and Tilly, 2004). As discussed in previous sections follicle selection is a highly regulated process and follicle atresia accounts for the substantial loss of many recruited but not selected follicles over the reproductive lifespan. It has been thought that, depending on the stage of follicle development, the cell type responsible for the initiation of atresia alters. The oocyte appears to initiate atresia in the resting or primordial follicles (Gougeon, 1996; Perez et al., 1999) whereas the initiation of apoptosis is transferred to the granulosa cells in later stages of development at the critical point of ovulatory selection (Hughes and Gorospe, 1991; Tilly et al., 1991; Johnson, 2003). It is important to acknowledge that all follicles are not alike. Both the susceptibility to apoptosis and the regulators of follicle survival change during each stage of development determining the fate of follicles. Furthermore, it has been demonstrated that follicle atresia occurs in conjunction with proliferation, with a higher incidence of follicle atresia observed in larger proliferating gonadotrophin-dependent follicles compared to small slow growth gonadotrophin-independent follicle.

Apoptosis permits the safe disposal of cells at the point in time when they have fulfilled their biological function since this process can be completed within minutes in single cells. However the breakdown and disappearance of a whole follicle takes several days depending on the size (Hussein, 2005). As discussed apoptosis is central to many functional aspects of the ovary with many molecular pathways involved, of which the Bcl-2 family, caspases and members of the TGFβ family play imperative roles. The recent advancements in molecular biology and the use of transgenic models has been invaluable in contributing to our understanding of ovarian apoptosis, but as a result of the complexity of the interactions follicular apoptosis remains only partly understood. There is a plethora of molecular mechanisms thought to be associated with the ovarian dynamics of atresia, with new molecular candidate being continually identified. Furthermore as a result of studies within this thesis a new novel molecule, PDCD4, has been identified to play a role in ovarian apoptotic mechanism but will be subsequently discussed in detail.

Molecules from both the intrinsic and extrinsic apoptotic signalling pathways and their potential roles in ovarian-related cell death are now discussed.

1.4.2. Caspase

Caspases are proteases, both initiator caspases (8 and 9) and effector caspases (3, 6 and 7) are required for activation of the caspase cascade. On activation of the caspase cascade, enzymes and proteins essential for cell viability are targeted and are cleaved, resulting in cell degradation and death. Caspases are activated in two ways in the granulosa cells: (i) cell surface receptors; and (ii) by members of the Bcl-2 family of proteins. Several members of the caspase family namely, 2, 3, 9, 11 and 12 have been directly implicated in mediating apoptosis within the ovary using genetic models of these caspases (Bergeron et al., 1998; Matikainen et al., 2001; Morita et al., 2001).

Inactivation of caspase 2 gene in mouse embryonic stem cells results in a marked decrease in developmental germ cell death resulting in an excess number of germ cells. Consequently, a significantly greater number of primordial follicles are present postnatally when compared to the wild-type controls (Bergeron et al., 1998). Alternatively in the absence of caspase 11 female mice are born with a severe depletion of germ cells, suggesting a different action compared to caspase 2 (Morita et al., 2001). However, it has been confirmed from experiments using caspase 11: caspase 2 double mutants that the germ cell loss observed in caspase 11-deficient females was dependent upon functional caspase 2 activity (Morita et al., 2001). Moreover, ovaries from caspase 9 deficient mice contain large numbers of developing follicles and as a result of apparent failure of granulosa cell apoptosis the entire follicle fails to complete atresia (Bergeron et al., 1998). The effector caspase, caspase 3 is present in its inactive (unprocessed) form within granulosa cells of healthy follicles, with atretic follicles possessing increasing concentrations of the cleaved activated form (Boone and Tsang, 1998). Furthermore the caspase 3 KO mouse model has demonstrated that the normal progression of apoptosis in granulosa cells from preantral to preovulatory follicles is dependent upon the activity of caspases 3 and 7 (Matikainen et al., 2001). Abnormal atretic follicles containing

granulosa cells that failed to be eliminated by apoptosis were detected in the caspase 3 mutant. In addition oocyte death whether initiated as a result of developmental cues or pathological insults was unaffected in the absence of caspase 3 (Matikainen et al., 2001). Ultimately these findings reinforces the conclusion that there are cell-specific apoptotic pathways present within the ovary and suggests that the paracrine–autocrine signals that initiate germ cell versus somatic cell death may differ.

1.4.3. Bcl-2 family

The B cell/lymphoma-2 family (Bcl-2) of proteins have been recognised as central contributors involved in the execution and control of regulated apoptosis (Adams and Cory, 1998; Chao and Korsmeyer, 1998; Minn et al., 1998; Reed, 1998). Both pro-apoptotic (Bax, Bcl-x_S, Bad, Bak, Bid, Bik/Blk, Bim, Hrk, Bok/Mtd) and anti-apoptotic (Bcl-2, Bcl-x_L, Mcl-1, A1, Bcl-w, NR-13Bax, Bcl-2-associated x protein) molecules have been identified. Their actions include forming checkpoints between factors on the cell surface and internal death signals, which then activate the required caspase cascade accordingly (Sato et al., 1994). An example of the integration of the apoptotic mechanism can be demonstrated by the mitochondria mediated apoptotic event which uses the death signals to cause the pro-apoptotic Bcl-2 proteins (especially Bax) to allow cytochrome c to leak out of the mitochnodria. This cytocrome c from the mitochondria in addition to the apoptotic protease activating factor 1 (Apaf-1) subsequently bind to caspase 9, activating the caspase cascade, resulting in cell death (Figure 1.8) (Morita and Tilly, 1999). It has been suggested from the studies to date that the fate of any given follicle whether it be atretic degradation or developmental survival ending in ovulating is ultimately determined by the end-result of a complex interaction between multiple Bcl-2 family members (Kim and Tilly, 2004).

1.4.3.1. Pro-apoptotic Bax

Bax is a pro-apoptotic factor demonstrated to be actively involved in follicle atresia and is the most studied member of the Bcl-2 family to date (Kim and Tilly, 2004). It has been demonstrated that apoptosis within the fetal ovary is correlated with an increase in Bax expression, with no observed difference in the expression of Bcl-2

(Felici et al., 1999). Additionally an increase in Bax expression at both the mRNA and protein level in a number of species has been demonstrated to be consistently correlated with granulosa cell demise and follicle atresia (Tilly et al., 1995; Gebauer et al., 1999; Zwain et al., 2001; Vitale et al., 2002; Yoon et al., 2002). Collectively these findings suggest that Bax is probably involved in multiple apoptotic-initiated pathways, both in the oocyte and granulosa cells.

Evidence for the physiological function of Bax has been provided by the generation of the Bax-deficient mouse. One of the major observations from this model is the accumulation of unusual atretic follicles containing numerous atrophic granulosa cells that presumably failed to undergo apoptosis (Knudson et al., 1995). Subsequent studies have additionally highlighted that the Bax deficient mouse displays a significantly reduced incidence of immature follicle atresia due to defective oocyte apoptosis postnatally, consequently prolonging the reproductive lifespan of these mice (Perez et al., 1999).

1.4.3.2. Anti-apoptotic Bcl-2

The major participant as a pro-survival molecule in apoptotic events is Bcl-2 classified as a proto-oncogene preventing apoptosis and has been demonstrated to play a role within the ovary. In mice a deficiency of Bcl-2 leads to the formation of a smaller cohort of resting primordial follicles (Ratts et al., 1995). Although unclear it has been suggested that this reduced cohort may result from an increase in apoptosis of germ cells either in fetal life, postnatally or, possibly, a combination of both (Kim and Tilly, 2004). In comparison the over-expression of Bcl-2 within the ovary results in an increase in ovulation rate, an indication of apoptotic down-regulation (Hsu et al., 1996).

1.5. Transgenic mouse technology

The use of transgenic mouse models have been repeatedly acknowledged and discussed in many parts of this descriptive review surrounding ovarian development. The use of this invaluable biological tool has highlighted the actions of many

individual genes and a remarkable magnitude of data is now in place regarding many of the key players in ovarian development. Thus, mouse genetics have provided unique insights into the function of multiple genes involved in the processes associated with oocyte growth and follicle maturation and will continue to do so in the future (Amleh and Dean, 2002). Many genetically modified mice now exist, either produced spontaneously or by design which show impairments in reproductive function and performance (Matzuk and Lamb, 2002; Rajkovic and Matzuk, 2002; Pangas and Matzuk, 2004). Consequently cellular and molecular characterisation of a number of these deficiencies has led to the identification of similar abnormalities in infertile humans (Vanderhyden 2002).

There are currently no oocyte cell lines, and transgenic animals remain the mainstay of research into the mechanisms of germline origin, differentiation and development. Mouse models represent invaluable tools to study *in vivo* genetics of mammalian oogenesis and to understand the mechanisms that lead to infertility at a molecular level (Pangas and Rajkovic, 2005). The DAZL mouse offers us a unique opportunity to address oocyte survival and intra-ovarian endocrinology and functionality (Ruggiu et al., 1997).

1.6. DAZL (Boule, DAZ)

The human DAZ gene was first detected after an investigation into azoospermia in infertile men, which is a condition whereby no sperm is present in the semen (Reijo et al., 1995). It has been identified that deletions within the Azoospermia Factor (AZF) region of the human Y chromosome may contribute to azzospermia, and of the deleted regions DAZ has been identified as a common mutation. DAZ is now considered essential for germ cell development and mutations or loss of function associated with infertility in the males (Lee et al., 1998; Ferlin et al., 1999;Kuo et al., 2004; Reynold and Cooke, 2005). Further investigations have led to the identification of the mouse autosomal homologue, DAZL (Cooke et al., 1995). DAZL is highly homologous to DAZ, with 83% similarity in the coding region of the cDNA, similar RNP/RRM (putative RNA-binding) domains and both of these genes

encode RNA binding proteins (Saxena et al., 1996; shan et al., 1996; Yen et al., 1996; Chai et al., 1997). Generally, RNA binding proteins play a fundamental role in the complex regulation of germ cell development, particularly in meiosis (Urano et al., 2005) and this complex process is subject to strict control at both levels of transcription and translation (Reynolds et al., 2005). A number of studies have identified specific mRNAs that are bound by the DAZ-related proteins *in vitro*, (Mains and Wasserman, 1999; Maegawa et al., 2002; Venables et al., 2001; Jiao et al., 2002; Urano et al 2005; Fox et al., 2005), with only a selected few showing true *in vivo* interactions (Reynolds et al., 2005, 2007), with focus remaining upon male spermatogenesis. Despite a number of potential target molecules having been identified in the testes their possible function within the ovary has not been investigated. Therefore identification of DAZL substrate mRNAs will now enable a detailed mechanistic assessment of the consequences of DAZL binding to target mRNAs within the ovary and to further elucidate the dose effect demonstrated by the Het females.

1.6.1. DAZL animal model

Most of what we know regarding the biology of DAZL and family members (Boule and DAZ) has come from studies conducted in non-mammalian vertebrates or male spermatogenesis. Ruggiu et al., (1997) were the first to confirm that the mouse DAZL gene was essential for development and survival of germ cells in both ovary and testis and from this initial work the molecular contribution to germ cell functionality has been explored. However, to date there has been little emphasis on the molecular function of DAZL within the ovary aside from the work previously accomplished in our lab (McNeilly et al., 2000; Elaine Watson, PhD, 2007). In the mouse it is now recognised through transgenic manipulation that in the absence of DAZL, germ cells fail to develop into follicles and are subsequently ablated from the ovary (Ruggiu et al., 1997), emphasising a fundamental role for DAZL in the processes involved in germ cell development and possibly follicle formation.

From the initial investigations utilising the transgenic DAZL model, the female phenotype was selected for further investigation to attempt to identify potential

DAZL mRNA targets. Organogenesis of the DAZL KO ovary appears normal, with a large number of pachytene cells present at e15. However there is a recognisable depletion in germ cell number by e19, with complete oocyte ablation postnatally (Figure 1.9) (Ruggiu et al., 1997). The KO females are therefore classified as completely infertile presenting with severely reduced or no follicular structures within the ovary. Furthermore the cells within their small ovaries are immunonegative for AMH and sulphated glycoprotein-1, an indication of the absence of small follicles and CLs consecutively (McNeilly et al., 2000). Interestingly, these ovaries show positive immunoexpression for 3β-hydroxysteroid hydrogenase, 17α-hydroxylase and aromatase, indicating the presence of steroidogenically active cells which are capable of producing oestrogen (McNeilly *et al.*, 2000). In addition the KO mice have estrogenic uteri supported by normal plasma concentrations of oestradiol (McNeilly et al., 2000). The investigations into the KO DAZL female phenotype are ongoing within our laboratory providing an interesting model for ovarian investigation in the absence of oocytes (McNeilly et al., 2000). An interesting observation was noted following breeding of these transgenic animals whereby the Het females produced significantly larger litters compared to the Wt (Figure 1.10) (McNeilly et al., SSR 2007) suggesting that their follicles may be more sensitive to FSH (McNeilly et al, 2000). The increased ovulation rate was associated with reduced levels of plasma FSH and increased levels of circulating inhibin B, suggesting that there may be more small follicles present or an increase inhibin B production from each follicle.

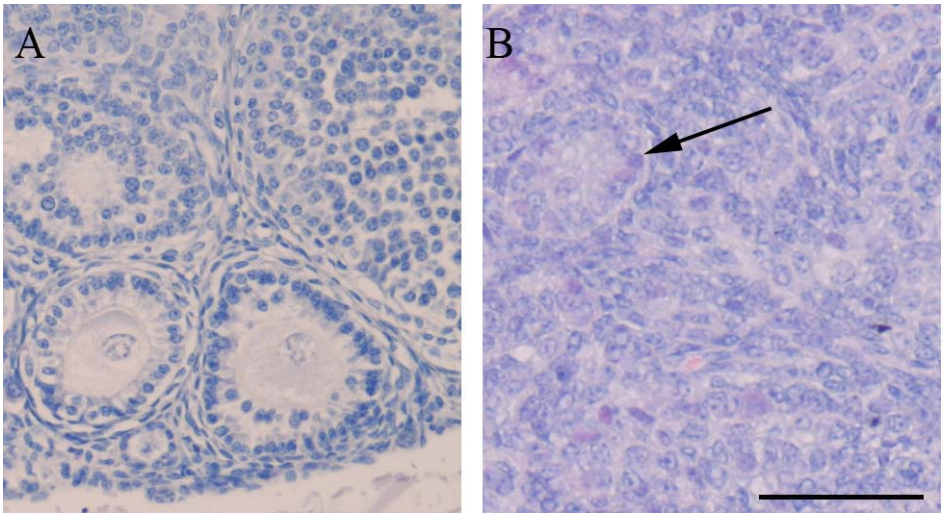

Figure 1.9: Histological photomicrographs of ovaries from d21 (A) Wt and (B) KO. Arrow indicates a follicle like structure in the KO ovary, but an absence of oocytes. Scale bar represent 100µm.

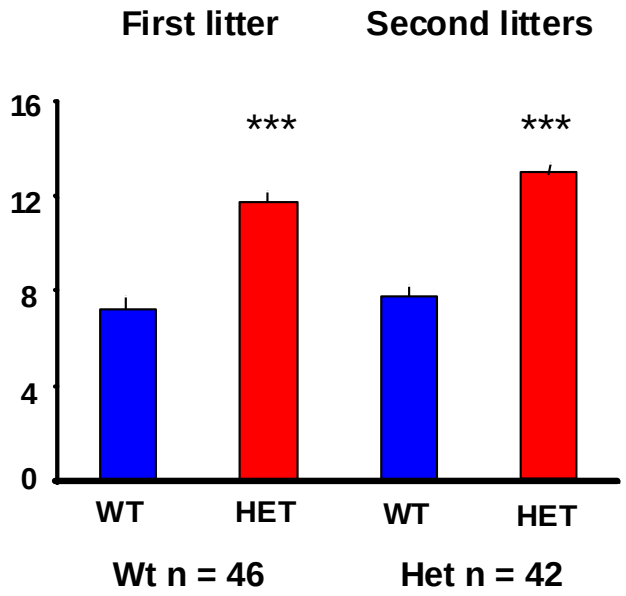

Figure 1.10: Histogram of Wt and Het litter sizes across two consecutive litters (Values are means ± SEM). *** denotes <0.001 significance (McNeilly et al., SSR 2007).

It has been proposed that since Het mice have elevated plasma inhibin B levels compared to the Wt, DAZL protein may not only have a direct functional role within the oocytes but functions indirectly on the capacity of granulosa cells to produce inhibin. Furthermore the increase in ovulation rate and subsequent litter size may be determined by either an increase in initial follicle recruitment, or enhanced function of individual follicles once selected into the growing cohort of follicles destined for dominance (McNeilly et al., SSR 2007). This increased selection may be due to an amplified sensitivity to FSH, thereby reducing follicle atresia of more sensitive follicles.

In addition previous work within our laboratory prior to the current studies assessed the ovarian follicle number in addition to follicle FSH sensitivity between DAZL Wt and Het female mice. Studies investigating whether follicle reserve was responsible for the increase in litter size observed in the Het females were performed with the ovarian follicle reserve from d21 pre-pubertal mice untreated mice, mice treated with 10iu FSH or ovine follicular Fluid (oFF) determined. Findings from these investigations conclude that untreated Het mice had a significantly greater percentage of antral follicles (<0.05) compared to Wt, however the difference was eliminated when endogenous FSH levels were suppressed by the oFF treatment or increased by exogenous FSH treatment, suggesting that FSH sensitivity is greater in the Het follicles compared to the Wt. In addition the antral follicles from the untreated Het mice had greater granulosa cell and follicle areas. Further studies investigating *in vivo* maturation of cultured follicles showed no difference in the endocrinology of Wt and Het follicles after culture with 1iu and 0.1iu FSH. However at 0.001iu FSH the Het follicles secreted higher levels of oestradiol ($p<0.001$) and inhibin A ($p<0.05$) suggesting greater maturity in the Het follicles (Watson, PhD 2007)

Furthermore after investigations comparing follicle health and atresia no difference in AMH (health) and cleaved caspase 3 (atresia) immunostaining between the Wt and Het in primary, secondary and antral follicles for untreated, FSH treated and oFF treated Wt and Het mice. However there were significantly more secondary follicles

from Het FSH treated mice which stained positively for AMH but were negative for cleaved caspase 3($p<0.01$) suggesting a higher proportion of healthy follicles compared to the Wt. Overall follicle activation from the reserve pool and atresia rates did not differ overall between Wt and Het follicles in untreated and oFF treated mice. However there was evidence of a greater activation or maintenance of healthy follicles after FSH treatment in Het mice

Moreover, evidence from these previous studies (McNeilly et al., 2000; Watson, PhD 2007) suggests that DAZL plays a fundamental role in the intra-ovarian communication between oocytes and somatic cells, sensitising the follicle to FSH. It can be suggested that activation, maintenance, sensitivity and maturity may all be consequently altered between the DAZL Wt and Het follicles. How this occurs has yet to be elucidated and despite a plethora of information regarding this RNA binding protein, the underlying functional mechanism and roles in ovarian development remain unidentified.

1.7. Thesis aims

Infertility or, more specifically, ovarian failure, arises as a result of many potential reasons, including extrinsic and intrinsic endocrine, steroidal or intra-ovarian control, ovarian pathology or disruption to the genetic control of the critically essential pathways involved in the complex developmental roles described above. There is emerging evidence pinpointing exact timings and essential roles for many factors with DAZL being a major candidate critical for oocyte survival.

This review has focused and highlighted a number of ovarian specific events and the roles that selected molecules play. It has highlighted the complexity of the processes involved in oogenesis and folliculogenesis. Investigating the functions of the germ cell specific DAZL will hopefully identify key pathways which may be targets to manipulate fertility and eventually enable scientist and clinicians to target problems associated with ovarian failure in humans.

The main aim for the experimental work detailed in this thesis was to further investigate how DAZL affects ovarian function, investigating why functional copy number (Wt and Het) of DAZL increases fertility and what physiological function does DAZL have on oocyte development and follicle function. To addresses these aims, firstly functional copy number was investigate to establish if DAZL expression contributed to an alteration in oocyte gene expression and hence regulatory control between the Wt and Het females (Chapter 3). Furthermore, direct and indirect approaches were adopted to identify potential DAZL binding mRNAs by *in silico* approach (Chapter 4) using the putative DAZL binding motif and by *in vivo* immunoprecipitation (Chapter 5). The emergence of one potential candidate was investigated further and identified a novel role in CL regression (Chapter 6). Finally *in vitro* follicle culture studies were utilised to investigate the intra-ovarian communication between the somatic cell and oocytes to further elucidate if DAZL is functioning directly or indirectly to affect oocyte and follicle development differently between the Wt and Het females (Chapter 7).

Chapter 2: General materials and methods

2.1. Animal experimentation

All studies were approved by the Home Office (United Kingdom) and performed under license number 60/3232 held by Professor Alan McNeilly in accordance with the Animals Scientific Procedures Act, 1986.

2.1.1. Animal accommodation

Experiments were conducted at the BRF (Biological Research Facility), Little France (Edinburgh) using DAZL Wt and Het female mice. Animals were housed and bred in a temperature (20-25°C) and light controlled room (14-h light, 10-h dark photoperiod) with food (standard laboratory CHOW) and water provided *ad libitum*. Daily management and care of the animals was carried out by animal technician Mark Fisken (MRC).

2.1.2. DAZL genotype

In all studies the female mice used were from our established colony of in-house breeding pairs (BRF) originally generated by a conventional knockout approach (Ruggiu et al., 1997). Heterozygous matings were subsequently used to provide offspring of mixed genotype.

2.1.3. Sacrifice and disposal of animals

Mice were euthanized at d10, d21 or adulthood by CO_2 inhalation within a sealed chamber followed by cervical dislocation in accordance with Schedule 1 of the Home Office regulations. Animal carcases were disposed of using the correct Health and Safety regulation in accordance to animal tissue and incinerated clinical waste.

2.2. Tissue collection

2.2.1. Ovary dissection

The abdomens of the mice and surrounding bench were sprayed with 70% ethanol prior to dissection to eliminate any possibility of introducing infection. The abdominal cavity of each mouse was carefully opened with a longitudinal incision using sterile autoclaved dissection scissors and forceps. The ovaries were located, carefully removed and any surrounding visceral fat tissue and attached oviduct were disconnected before the ovaries were either:

- Snap frozen on dry ice for subsequent messenger RNA (mRNA) analysis or protein extraction (Section 2.3; 2.6).
- Fixed in Bouins (2 hours) or 4% Neutral Buffered Formalin (NBF) (24 hours) before being transferred into 70% ethanol for paraffin imbedding (Section 2.7).
- Placed in HANKS (balanced salt solution modified, with sodium bicarbonate, without phenol red, calcium chloride and magnesium sulfate, liquid, sterile-filtered, cell culture tested) (Sigma, Aldrich, UK) for further microdissection.
- Placed in warmed L-15 medium (Sigma) for follicle dissection (Section 7.2).

2.2.2. Oocytes

Oocytes were manually dissected from follicles in HANKS solution using 27G needles (BD microlance) attached to 1ml syringes (BD Plastipak, Spain). Follicles were punctured to release the oocyte and any surrounding granulosa cells were removed from these oocytes by repeated pipetting before being collected using a capillary tube and mouth pipette (Sigma), under a dissection microscope (Leica). Collected oocytes were the snap frozen on dry ice for subsequent RNA analysis and PCR.

2.2.3. Follicles

Intact follicles were manually dissected from the ovary by carefully teasing the ovary apart taking great care not to disrupt the basement membrane between adjacent follicles. Follicles of different sizes were selected using a calibrated graticule in the

eye piece of the dissection microscope (taking into account the distortion produced by the contours of the embryo dishes in which the dissections were taking place). Follicles were either selected on size for RNA extraction or for follicle culture (Section 7.2).

2.2.4. Tissues

Tissue samples of testis, spleen, heart, liver, kidney, lung and adrenal were located and dissected, snap frozen on dry ice for further RNA or protein extraction or fixed in Bouins or NBF. These tissues were used for subsequent tissue screens or as control tissues.

2.3. Ribonucleic acid (RNA) extraction

2.3.1. Guidelines for the use of RNA

It is careful to note that RNA molecules are subject to nuclease digestion by RNases, which act by catalysing and hydrolysing RNA into smaller components. RNases are present on the skin and in the atmosphere therefore it is essential that gloves were used at all times. Equipment was cleaned and RNase-free filtered tips, plastics and solutions were used.

2.3.2. Extraction

RNA is comprised of three component subtypes representing the processes of translation and transcription: messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). Total RNA was extracted using either mini or micro RNAeasy® Kits (Qiagen) following the total RNA isolation from the animal tissue manufacturer's protocol. The micro kit allows reliable RNA isolation from small samples, such as oocytes whereas the mini kit is more suited to larger tissue samples such as whole ovary or testis. Carrier poly-A RNA was added to samples of low yielding properties at the time of lysis at 4ng/µl. The addition of small amounts of poly-A RNA used as carrier RNA do not interfere with subsequent RT-PCR, even when oligo-dT is used as a primer for reverse transcription.

Briefly, samples were lysed using 350µl of supplied RT buffer supplemented with 1% β-Mercaptoethanol (Sigma) and homogenised using a hand held homogeniser (Sigma-Aldrich). 350µl of ethanol (VWR) was added to the tissue lysate to provide ideal binding conditions for the RNA prior to the sample being loaded onto the RNeasy MinElute spin column. The RNA binds to the silica-gel membrane and traces of DNA that may copurify were removed by a DNase treatment (Qiagen) on the RNeasy spin column. The DNase treatment was applied to the column and left on the bench at room temperature for 15 minutes. The DNase and any contaminants are efficiently washed away by a series of washes using supplied solution RW1 (350µl), the column was dried using 80% ethanol, and pure, concentrated RNA was eluted from the column in 14µl of water.

2.3.3. Assessment of RNA

RNA quantity was assessed using a NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). When using the nanodrop 1.5ul of sample is required and the absorbance is measured at 260 and 280 nm. The sample is pipetted onto the end of a fibre optic cable (the receiving fibre), a second fibre optic cable (the source fibre) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the fibre optic ends. The gap is controlled to both 1mm and 0.2mm paths. A pulsed xenon flash lamp provides the light source and a spectrometer utilising a linear CCD array is used to analyse the light after passing through the sample. The instrument is controlled by special software run from a PC, and the data is logged in an archive file on the PC (Figure 2.1). The nucleic acid concentration is automatically calculated using the Beer-Lambert law (Equation 2.1), which predicts a linear change in absorbance with concentration. The ratio of absorbance at 260 and 280 nm is used to assess the purity of RNA. A ratio of 1.8-2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

Beer-Lambert law Equation

$$A=\varepsilon CI$$

A=absorbance at a particular wavelenghth

C= concentration of nucleic acid

I= path length of the spectrophotometer cuvette (typically 1cm)

ε= the extinction coefficient (ε for RNA is 0.025 (mg/ml)

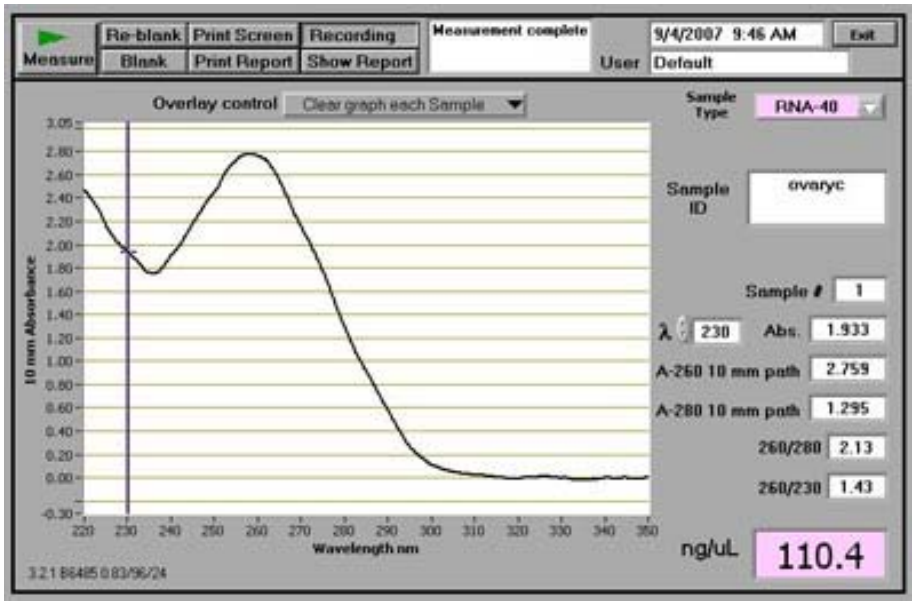

Figure 2.1: Output data from the nanodrop showing the 260:280 ratio, absorbance peak and the concentration of RNA in the sample.

2.3.4. Reverse transcriptase polymerase chain reaction

The polymerase chain reaction (PCR) is an extensively used molecular technique for the rapid and efficient amplification of cDNA reverse transcription from RNA to quantify gene expression. For samples containing <50ng RNA complementary DNA (cDNA) was prepared using Sensiscript® Reverse Transcription for first-strand cDNA synthesis (Qiagen), while for samples containing >50ng RNA Omniscript Reverse transcription was used. The reaction components are listed in Table 2.1 and

once made the reaction was incubated for 60min at 37°C using PTC200 (Peltier Thermal Cycler) PCR block.

Table 2.1: Reaction components for Qiagen sensiscript cDNA synthesis

Reagent Master Mix	***X 1 (µl)***	**Final Concentration**
10x RT Buffer	2	**1x**
dNTP mix 5mM each (dNTP)	2	**0.5mM each dNTP**
Oligo dT (10µM)	0.4*	**1 µM**
Sensiscript/Omniscript RT	1**	**10 units per reaction**
RNase free H2O	variable	
Template (RNA)	variable	**<50ng (per reaction Sensiscript)** **>50ng (per reaction Omniscript)**
Total	**20**	

* Taqman concentration

**diluted 1:1 with 1x RT Buffer

2.3.5. Oligo dT and random hexamer primer choice

Most cDNA reactions used oligo (dT) primers which anneal selectively on the poly(A) tail of mRNA. The use of oligo (dT) reduces the cDNA complexity and amount since poly A+ RNA represents only 1%-2% of total RNA compared to random hexamer primers which can be used for transcription of the 5'-end regions of mRNA. However, oligo (dT)s are recommended when performing RT-PCR of a new mRNA targets and produce RT-PCR products more consistently than random hexamers. Random hexamers are required to be used when performing Taqman probe/primers PCR reactions.

2.3.6. PCR reaction

All standard PCR reactions were performed using Qiagen HotStartTaq DNA Polymerase. HotStartTaq is supplied in an inactive state that has no polymerase activity at ambient temperatures thus preventing extension of non-specifically annealed primers and primer–dimers which may form at low temperatures during

PCR setup and the initial PCR cycle. The PCR reaction is a thermoregulated cyclic process whereby the double stranded cDNA is separated, specific primers annealed to the gene of interest and newly synthesised DNA is formed (Figure 2.2).

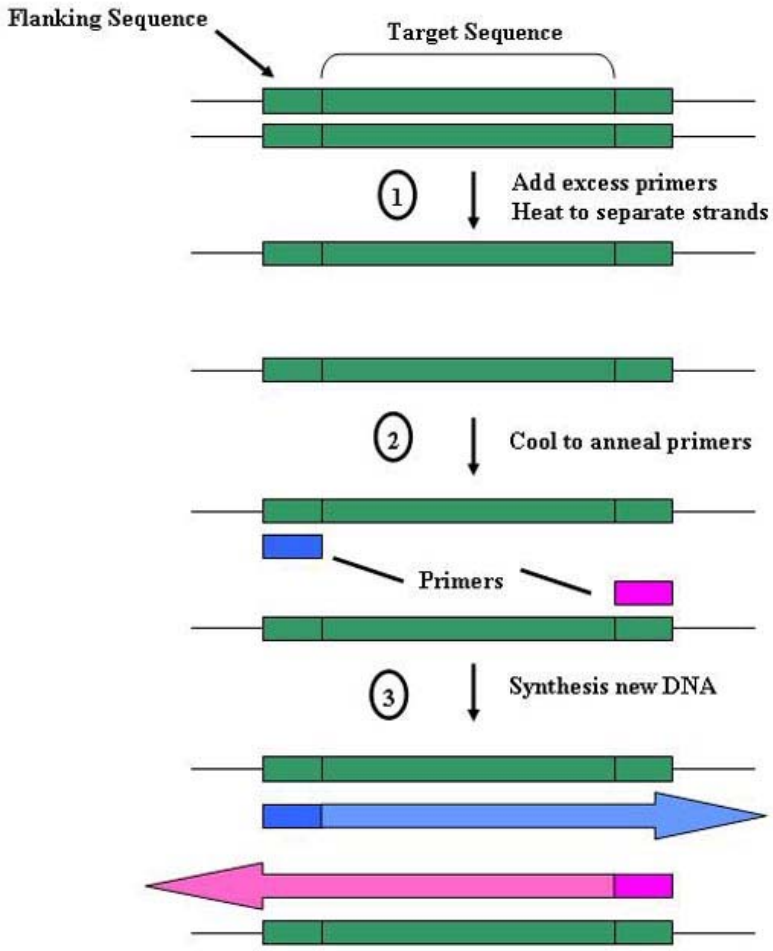

Figure 2.2: The first cycle of a polymerase Chain Reaction (PCR) A cycle consists of three steps: strand separation, hybridization of primers, and extension of primers by DNA synthesis.

The components of the PCR reaction are shown in Table 2.2 (for negative controls the reverse transcriptase was omitted), reactions were incubated using the PTC200 (Peltier Thermal Cycler) PCR block using the following conditions:

Initial activation step:	95°C for 15 minutes
3 step cycling	
Denaturation:	94°C for 1 minute
Annealing:	60°C for 45 seconds
Extension:	72°C for 1 minute
Number of cycles:	34 cycles
Final extension:	72°C for 10 minutes

Table 2.2: Reaction components for Qiagen Hotstart Taq Polymerase

Master Mix	***X 1 (µl)***
10 x buffer	1
25µm MgCl2	0.6
ddH2O	6.62
20µm Primer 3'	0.25
20µm Primer 5'	0.25
10 mM dNTPs	0.2
Qiagen Hotstart Taq	0.08
Template	1
Total	**10**

2.3.7. Agarose gel electrophoresis

After the PCR reaction, separation of DNA product molecules on agarose gels (Section 2.13) allowed visual confirmation of DNA size and concentration. TBE buffer (45mM Tris borate, 1mM EDTA pH8) was used for high resolution of DNA fragments typically less than 500bp in length. Ethidium bromide (Sigma-Aldrich, UK) and latterly "Safeview" (NBS Biologicals) were used as visualising agents and were added to the gel at a final concentration of 5ug/ml. 6x loading buffer (Promega) was used to visualise the loading of the PCR product sample and increase the density of the sample. Products from the PCR reactions were compared with 100bp molecular markers (Promega, UK) to check they were the correct amplified

size. A voltage of 100 volts was applied to the gel for 20 minutes (Bio-Rad Power Pack). Visualisation of DNA in the presence of the fluorescent intercalculating agents was undertaken using an ultraviolet transilluminator (302um wavelength) and photographed using an integrated camera (Gene Flash, Syngene, Bio Imaging).

2.3.8. Oligonucleotide primer design

Oligonucleotides were designed specifically for use in RT-PCR and SYBR green real time RT-PCR using a combination of Primer 3 and MWG-biotech software. When designing the requisite pair of specific oligonucleotide primers several guidelines were used in the selection process. The length of the primer molecule was constrained to between 18 and 22 bases with a melting temperature (Tm) between 55 and 65 °C where possible using the calculation below:

Tm = (G+C x4°C)+(A+T x2°C)

G = guanine
C = cytosine
A= adenine
T = thymine

Secondary structure formation was checked for avoiding the occurrence of hairpin loop formation and intra-molecular annealing. Finally, for quantitative Real Time PCR the product length was controlled to 100-250 base pairs for an optimal reaction.

2.4. Real time semi quantitative RT PCR

2.4.1. Light cycler (Roche)

Quantitative PCR was carried out using QuantiTect™ SYBR® Green PCR (Qiagen) on the Roche Light Cycler system (Roche). SYBR Green dye binds to all double-stranded DNA, and thus any nonspecific products and primer–dimers formed during real-time PCR will negatively affect quantification of the target gene. The QuantiTect SYBR Green PCR Kit overcomes this problem with a unique PCR buffer that promotes highly specific annealing of primers to the PCR template. In addition, the buffer contains HotStarTaq DNA Polymerase, which provides the most stringent

hot start compared with other polymerases. The unique composition of the RT-PCR buffer and HotStarTaq DNA Polymerase enable quantification of even low-abundance transcripts with as low as 5 copies of a target accurately detected (Qiagen). The master mix (Table 2.3) was aliquoted into each capillary at a volume of 9µl and 1µl of cDNA from each sample diluted 1:2 was added to the master mix. Sample were analysed in triplicate.

Capillaries were centrifuged at 0.6 rpm for 10 seconds to ensure mixing of sample and master mix, before being loaded carefully into the light cycler carousel. Once the run was complete the PCR product was run on 2% agarose gel to confirm amplification of a single product of the correct size.

Table 2.3: Reaction components of SYBR green real time PCR

Component	***X 1 (µl)***	***Final Concentration***
2x QuantiTect SYBR green PCR Mastermix*	5	1x
Primer A (20µM)	0.5	0.5 µM
Primer B (20µM)	0.5	0.5 µM
H2O	3	variable
Template	1	<1 µg/reaction
Total	**10**	

*Provides a final concentration of 2.5mM $MgCL_2$

Due to the limitations of the sample number (n=32) on the light cycler, the SYBR green reaction was transferred to the Taqman machine (ABI PRISM 7900HT), where 96 samples can be analysed at the same time.

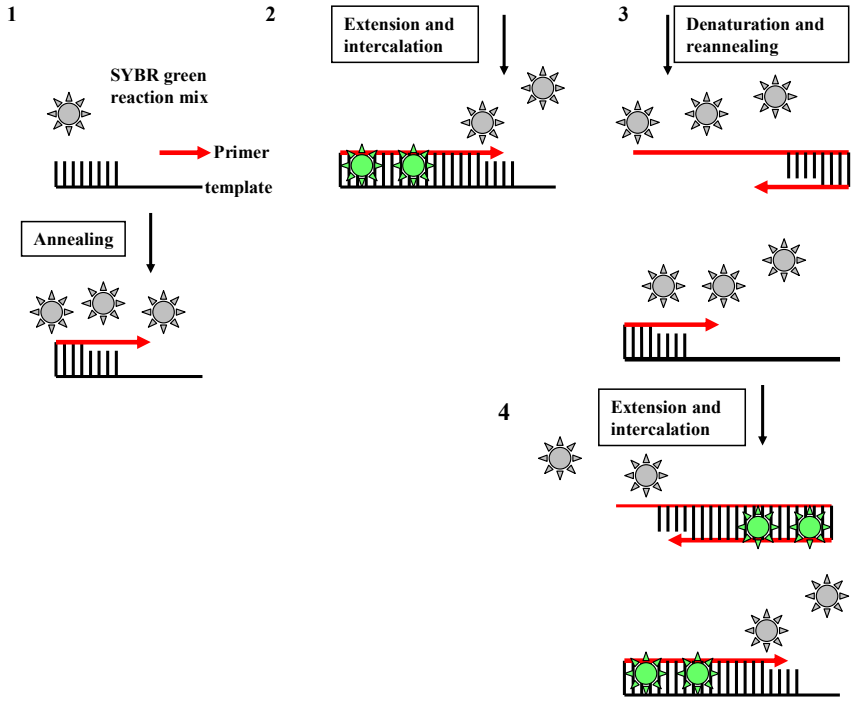

Figure 2.3: Diagram of how fluorescent SYBR green incorporates into the PCR reaction 1: annealing, 2: extension and intercalation, 3: denaturation and reannealing 4: further extending and intercalation of SYBR green (adapted from http://www.sigmaaldrich .com/img/assets/9421/PCR-Quant_PCR.pdf).

2.4.2. Taqman SYBR Green

Using Optical 96-well fast thermal cycling Plate with Barcode (code 128) and QuantiTect™ SYBR® Green PCR (Qiagen) 96 reactions can take place at one time. The reaction master mixture (Table 2.3) was aliquoted for each sample into separate plate wells with cDNA added at 1µl/10 µl reaction mix and again each sample was run in triplicate. An optical adhesive cover was used to seal the plate and the PCR reaction was then run on the ABI PRISM 7900HT (Applied Biosystems) using optimised fast conditions (Figure 2.4).

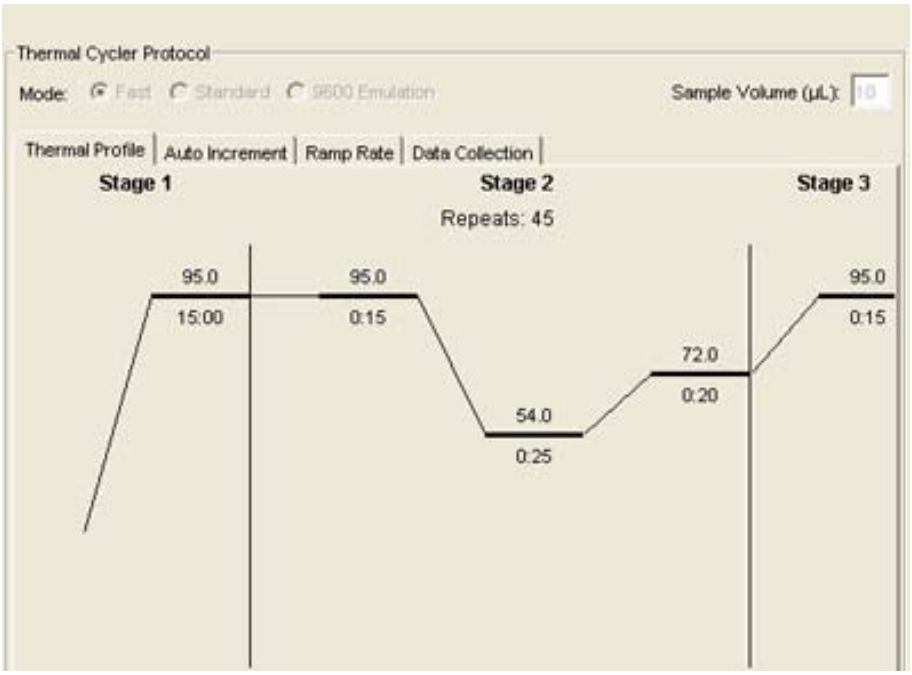

Figure 2.4: Thermal Cycle protocol for SYBR green reactions.

2.4.3. Primer validation and Analysis

Accurate quantification of gene expression levels requires a standard curve to validate the primers. To generate this standard curve, a dilution series was made from a cDNA sample. Real-time PCR assays were carried out in triplicate in order to minimise the contribution of pipetting and PCR variation. In addition to the samples to be quantified and the dilution series for the standard curve, mock reactions lacking template as a control were also used to verify that there was no potential contamination of any solution with PCR product, which is always a concern with any PCR-based procedure.

The Applied Biosystems software collects fluorescence data throughout the PCR cycle and automatically analyses baseline and amplification parameters. Baseline fluorescent intensity is subtracted from each analysed PCR samples, and an amplification plot is generated. Using data from samples designated as standard points with assigned concentration numbers, an arbitrary threshold level is set and Ct values for all PCR samples were calculated, allowing generation of a standard curve and corresponding correlation coefficients (R^2) and slope (PCR efficiency). A high-

quality standard curve should have an R^2 greater than 0.995 and PCR efficiency between 90 and 100%. Based on this standard curve, the concentrations of all unknown samples can be derived using the equation of the graph of the standard curve. Averages were taken among replicates. Generally, this automatic analysis of real-time raw fluorescent data is sufficient and was used in the current studies. In the experiments carried out in this thesis normalization of data was performed assuming equal efficiency of cDNA synthesis, imput cDNA and efficiency of PCR reaction and results were obtained by using the standard curve quantification.

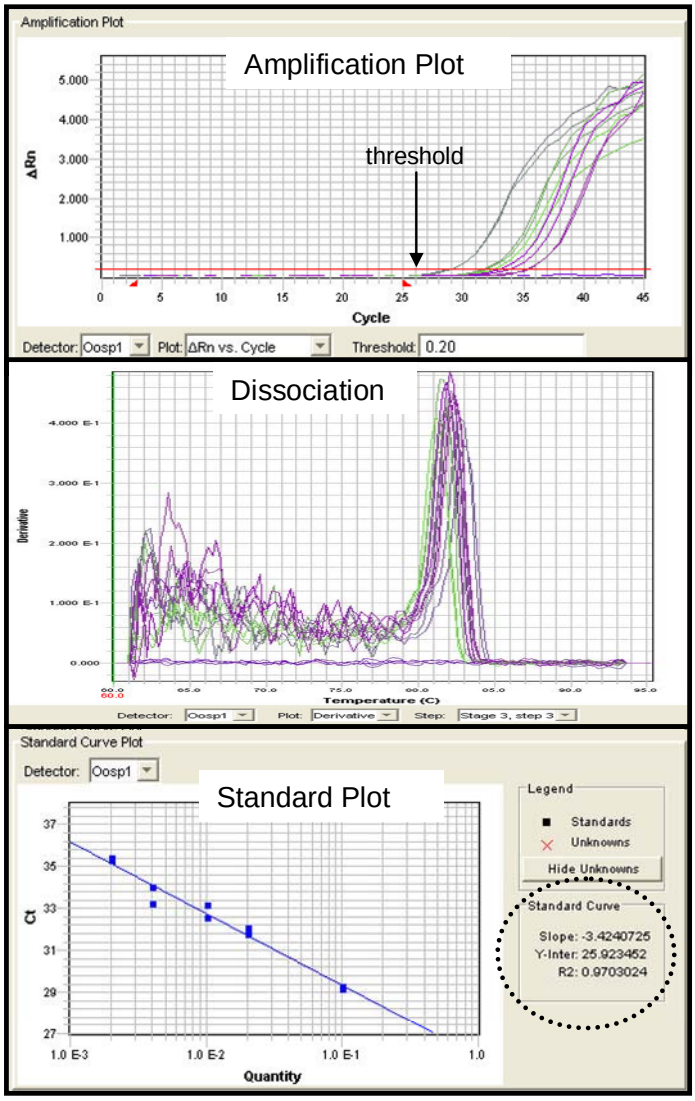

Figure 2.5: SYBR green analysis, amplification plot, dissociation curse and standard plot highlighting the R2 value and slope efficiency.

2.4.4. Advantages and disadvantages of SYBR green PCR

There are advantages and disadvantages in using the SYBR green PCR reaction over Taqman primer probe analysis. SYBR green can be used with specific primers and can be used to monitor the amplification of any double-stranded DNA sequence without the requirement of a probe which may reduce assay setup and running costs. However the primary disadvantage of the SYBR Green I dye chemistry is that it can generate false positive signals because SYBR Green I dye can bind to any double-stranded DNA. Thus non-specific double-stranded DNA sequences may occur as a result of primer dimer interactions.

2.4.5. Taqman quantitative RT-PCR

Quantitative PCR was also performed using the Assay-On-Demand Gene Expression™ system (Applied Biosystems) on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The Taqman® Assay-on-Demand probes have a FAM™ reporter dye at the 5' end of the Taqman MGB (minor groove binding) probe and a non-fluorescent quencher dye at the 3' end.

2.4.5.1.Principles of the Taqman reaction

Briefly, cleavage of the probe separates the reporter dye from the quencher dye, which results in increased fluorescence of the reporter. This only takes place if the specific probe has hybridised to the target sequence of cDNA. Accumulation of the PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye (Figure 2.6). The expression level of the gene of interest was related to an endogenous control, 18S ribosomal RNA and negative controls were included in each run. The uses of an internal reporter primers increases the specificity of quantification of the PCR reaction compared with the SYBR green method, but analysis of the same sample by both methods gave similar results.

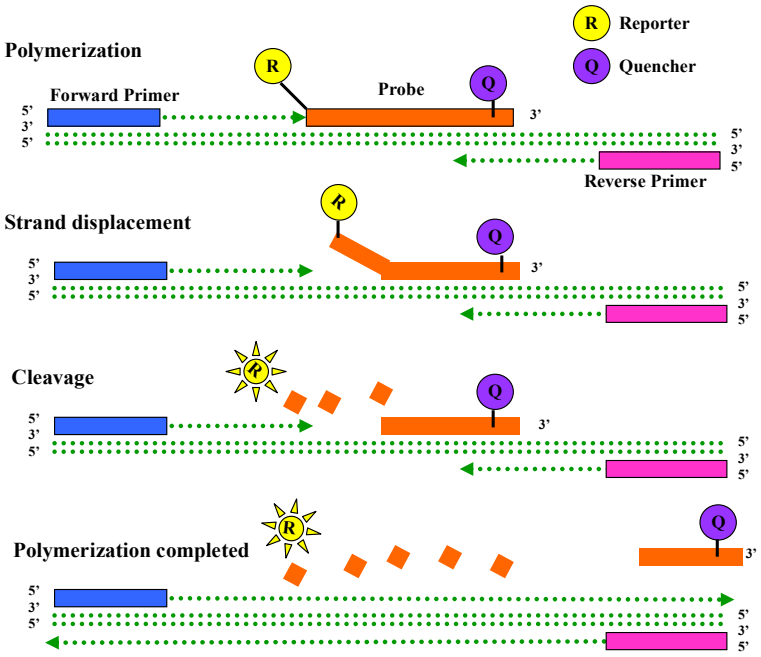

Figure 2.6: Schematic diagram showing polymerisation, strand displacement, cleavage, and completed polymerisation steps in the Taqman PCR reaction (adapted from Applied Biosystems, Chemistry guide, online).

All reagents were obtained from Applied Biosystems and samples run in triplicate on 96 well MicroAmp Fast Optical reaction plates (Applied Biosystems). For each sample the PCR reaction was prepared to give a total volume of 10µl in each well and the plate was sealed with an adhesive cover (Applied Biosystems).

2.4.5.2.Primers

Primer and probe sequences were either designed using Primer Express software and synthesized by PE Biosystems or by Biosource Europe SA (Nivelles, Belgium) by Linda Nicol (FST, inhibin βB, AR11a, LHR and FSHR) or were supplied assay on demand by PB biosystems (aromatase, inhibin α, inhibin βA and AMH). Ribosomal 18S primers and probe were from a Taqman Ribosomal RNA Control Reagents kit (VIC labelled probe) (Table 2.4).

Table 2.4: Sequences of primers and probes used for Taqman RT-PCR analysis

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	Probe (FAM labeled)
LHR	ttttatcctgtcaattcttgtgccaaccca	ccttcgtgaacactgcgtaca	actgtcaccaactcaaaagttctg
FSHR	gcctgcccatggatatcg	aggccagggcgttgagtac	ctttgtcgcagctgtatgttatggccctc
Inhibin βB	atgtcccctgccagttca	ctcaggagggaaagtgcattg	ttgccctcccgcctgctcc
AR11a	aagttcgaggctggcaagtc	caacacctctggagccatatacct	cgggtaccaacctgccatggg
FST	gggctggatgggaaaaccta	cggctgctctttgcatctg	cgcaacgaatgtgcactcctcaagg
	Assay on Demand		
Aromatase	Mm00484049_m1		
AMH	Mm00431795_m1		
Inhibin α	Mm00439682_m1		
Inhibin βA	Mm00434338_m1		

2.4.6. Analysis

Absolute quantification was performed on the ABI Prism sequence detection system using Real Time PCR. Real Time PCR reactions are characterised by the point in time during cycling when amplification of a target is first detected rather than by the amount of target accumulated at the end. The earlier the target is detected the more cDNA is present.

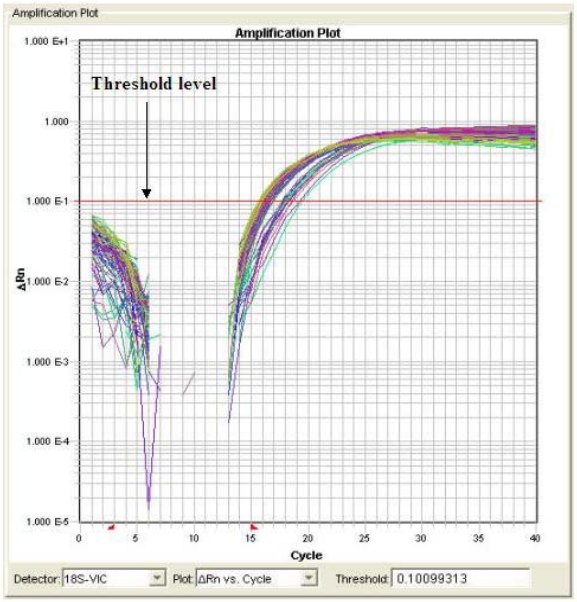

Figure 2.7: Example of an amplification plot after a successful Taqman RT-PCR reaction.

The amplification plot produced from the Taqman PCR shows the amount of reporter dye generated during the amplification stage (Figure 2.7). This amplification is directly related to the amount of PCR product formed, which in turn is related to the expression level of the target gene. The FAM Ct (threshold cycle) value corresponds to the cycle number at which the fluorescence generated crosses the threshold level. The threshold level is defined as the point at which an increase in signal is associated with an exponential increase of PCR product. The Ct value is directly related to the amount of PCR product with a change in Ct value of one equating to a two-fold difference in initial cDNA concentration.

Analysis of the results was carried out using the comparative Ct method. This is used to analyse changes in expression of a target gene relative to another reference sample. For each sample, the ΔCt was calculated which refers to the difference between the FAM Ct (gene of interest) and the 18S Ct which normalises the amplified signal against the total mRNA content. The mean ΔCt for the triplicates was calculated and used to determine the ΔΔCt, which is the difference between the ΔCt of the experimental sample compared to that of the control or untreated sample. The amount of amplified target is given the value of 2- ΔΔCt which is based on the mathematical equation that describes the exponential amplification of the PCR reaction: Xn = Xo x (1+Ex)n where Xn is the number of target molecules at the threshold at cycle n, Xo is the initial number of target molecules, (1+Ex) is the efficiency of the target gene amplification and n equals the number of cycles. Assuming that the efficiencies of the target and endogenous control reactions are equal, the 2- ΔΔCt value is a measure of relative quantification and is used here to show the fold difference in mRNA expression of the samples relative to the control sample, where the control is given a 2- ΔΔCt of 1.

2.5. Genotyping

2.5.1. Digestion

Genotyping was carried out on tail tips collected at sacrifice of littermates to distinguish between DAZL genotypes. The genotyping PCR previously established

(MRC, HGU, Edinburgh) was performed on a crude digestion of the tail tips whereby 100ul of 25mM NaOH (BDH) 0.2mM EDTA (Digest 1) (Sigma) was added to each sample and digested for 20 minutes at 95°C (Omnigene, PCR machine). The reaction was centrifuged before the addition of 40mM Trizma HCL (Digest 2) to stop the digest reaction and the sample vortexed and centrifuged. The digest was then used for the genotyping PCR.

2.5.2. Genotyping PCR

The genotyping PCR was carried out using Abgene Hot Start Taq under optimal PCR conditions (Table 2.5) using specifically designed primers (Table 2.6). For each individual sample 50µl of master mix was added to a 0.2ml PCR tube, with 5µl of tail tip digest, dH20, Digest 1 or Digest 2 (as negative controls). The PCR reaction was performed as previously mentioned (Section 2.3.6) but in a final volume of 50µl. The gel was photographed using a UV transilluminator, where the top band represents Wt, bottom band KO and the presence of both bands Het genotypes (Figure 2.8).

Table 2.5: Reaction components for Genotyping PCR (Abgene, Epson, UK)

Master Mix	**X 1 (µl)**
10 x buffer	5
25µm MgCl2	4
ddH2O	39.1
20µm Primer u660/661	0.6
20µm Primer u662/663	0.6
10 mM dNTPs	0.5
Abgene Hotstart Taq	0.2
Total	**50**

Table 2.6: Primer sequences for DAZL genotyping

Primer	Forward 5'-3'	Reverse 5'-3'
U660/661	CAg Tgg CTT gAA ATT ATC	gCT TCC TCT TgC AAA ACC AC
U662/663	CCT CCT CCA CAg TTC CA	TgA TTT CAg CTT AgC ATA AAC AgC

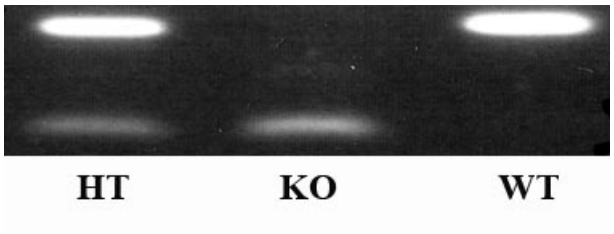

Figure 2.8: Photomicrograph of a PCR distinguishing between DAZL genotypes.

2.6. SDS PAGE/Western Blotting

SDS PAGE and western blot analysis can detect individual proteins of interest from a mixture of a great number of proteins. The Licor odyssey fluorescent detection system was used for all Westerns performed.

2.6.1. Protein extraction

Total protein was extracted from mouse tissues previously dissected (Section 2.2) and frozen (-70ºC) tissues using RIPA buffer (Section 2.13) containing a protease inhibitor cocktail (Roche). The tissue was homogenised in 50-200µl RIPA buffer using a handheld motor driven grinder (Sigma). The homogenate was centrifuged at 2500rpm for 10 minutes. The protein rich supernatant was collected in a fresh 1.5ml tube and stored at -70ºC until use.

2.6.2. Protein quantification

Protein samples were quantified in order to carry out comparative expression analysis. A Biorad DC protein assay kit (Biorad Laboratories, Hemel Hempstead, UK) was used to determine concentration according to the manufacturer's instructions. This kit is a colorimetric assay which is based on the reaction of the

protein with an alkaline copper tartrate solution and Folin Reagent. This assay is a two step process firstly involving the reaction between protein and copper in an alkaline medium, and secondly the reduction of the Folin reagent by the copper-treated protein. The colour analysed is mainly due to the amino acids tyrosine and tryptophan, and to a lesser extent cystine, cysteine and histadine. The reduction of Folin reagent produces a blue colour observed with a maximum absorbance at 750mm and a minimum at 405nm (Lowry et al., 1951 and Peterson and Gary 1979). Briefly, a standard curve was set up using solutions of known concentrations of BSA in RIPA buffer. These standards along with the samples of unknown concentration were subjected to a two-step assay based on the Bradford method of protein quantification. The samples are then read on a spectrophotometer plate reader (Labsystems Miltiskan EX; VWR) at 690nm. The concentration of each unknown sample was determined using the standard curve produced from the protein standards.

2.6.3. Sample preparation

Tissue samples previously quantified (Section 2.6.1) were diluted in PBS to the desired concentration and 5µl loading buffer added (Section 2.12). The samples were boiled for 5 min immediately before loading each protein sample into the wells on the gel, between 20-100µg of the denatured protein sample was loaded per well (see individual experiments).

2.6.4. SDS PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) is a technique used to separate protein according to their electrophoretic mobility and molecular weight. For each gel, 5µl of molecular weight marker (Odyssey) was loaded into at least one lane. This marker contains proteins of known molecular weight thus allowing identification of the target protein at the correct molecular weight and allows visualisation of the transfer of protein from gel to membrane. Protein samples were run on 10% acrylaminde gel (Section 2.12) (with 3% stacking gel) in an electrophoresis tank, containing SDS Running Buffer (Section 2.12), at 100V to resolve the proteins according to their molecular weight. Electrophoresis

was stopped when the bromophenol blue dye reached the bottom of the gel, approx 2 hours.

2.6.5. Transfer of proteins

To further analyse the protein, the glass plates were carefully separated allowing access to the gel which contains the separated proteins. The proteins were then transferred to a membrane by western blot analysis. The components of the blotting apparatus included two porous pads, 6 pieces of 3mm Whatman paper and nitrocellulose membrane (Immobilin-P; Millipore, Bedford, UK) which was rehydated in methanol for 30 seconds and then washed in transfer buffer prior to use (Figure 2.9). This arrangement allows the separated proteins to transfer from the gel towards the anode until they reach the solid support membrane. The chamber was filled with the transfer buffer and attached to a power pack for 1h at 100V. An ice pack was added to the chamber to prevent over heating as a result of the applied current and to allow optimal transfer conditions.

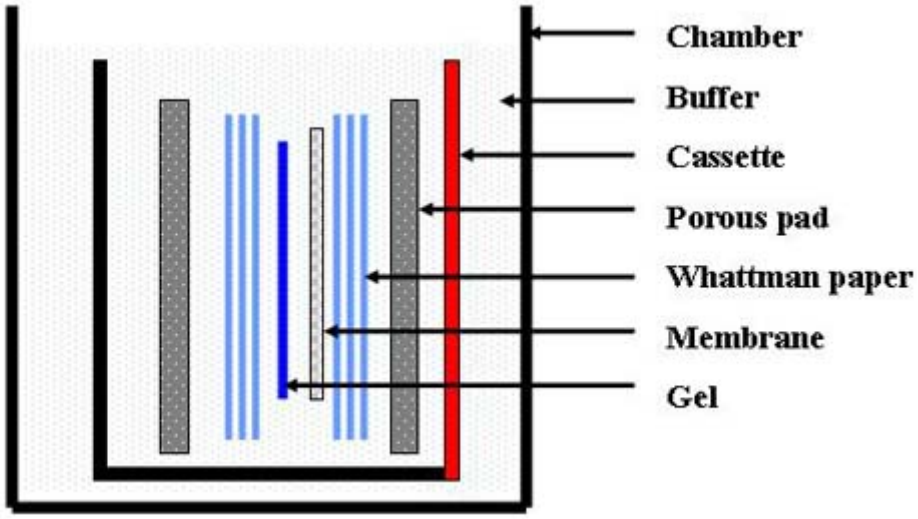

Figure 2.9: Western Blot transfer set up

2.6.6. Probing the membrane

Once transfer was complete the membrane was blocked using Odyssey® blocking buffer (LiCor, Nebraska, USA) diluted in PBS (1:1) and incubated for 1h at room temperature. This stage blocks any non-specific binding sites on the membrane.

2.6.6.1.Primary antibody

The membrane was subsequently incubated overnight with the primary antibody diluted in Odyssey® blocking buffer/PBS-Tween (PBST) at 4ºC. The antibodies and dilutions used are detailed in table (Table 2.7).

Table 2.7: Primary antibodies used in Western Blot analysis

Antibodies	Source	dilution
DAZL	Abcam	1:1000
B-tubulin	Abcam	1:1000

After primary antibody incubation the membrane was rinsed in PBST (4 x 5 minutes) so that any residual primary antibody was eliminated from the membrane.

2.6.6.2.Secondary antibody

The appropriate secondary antibody which were fluorescently labelled and directed against the host species of the primary antibody (Table 2.8) were diluted 1:5000 in the blocking buffer and incubated with the membrane for 1h at room temperature. Finally the membrane was washed in PBST (4 x 5 min) to remove excess antibody before analysis.

Table 2.8: Summary of secondary antibodies used on Western blots for LiCor detection

Fluorescent antibody	Species	Supplier	Excitation wavelength (nm)
Alexafluor®680	Donkey anti goat	Molecular Probes	680
IR Dye 800	Goat anti mouse	Rockland	800
Alexafluor®680	Goat anti mouse	Molecular Probes	680
Odessey 680	Goat anti mouse	Odessay	680

2.6.7. Protein expression analysis – LiCor technology

Protein expression was analysed on a LiCor fluorescence detection system (LiCor). The LiCor allows detection of two proteins simultaneously using different coloured

fluorescent markers and fluorescent filters. Membranes were washed in PBS alone to remove any residual Tween-20 prior to analysis.

2.7. Tissue fixation and processing

2.7.1. Tissue fixation

Tissues were either fixed in NBF (Section 2.12) for 24 hours or Bouins (Triangle Biomedical Sciences, Lancashire, UK) for two hours before being placed in 70% ethanol prior to tissue embedding. Subsequent immunohistochemisty used NBF fixed tissue unless otherwise stated.

2.7.2. Tissue processing and paraffin embedding

The tissue was processed and dehydrated through a series of graded alcohols on a 17.5h cycle using an automated Leica TP-1050 processor (Leica Microsystems, Milton Keynes, UK). Processed tissue was saturated and embedded by hand in liquid paraffin wax and cooled. The finished wax blocks were stored at room temperature prior to use.

2.7.3. Sectioning of paraffin blocks

5µm paraffin sections were cut using a hand-operated microtome (RM2135; Leica, Germany) from chilled wax blocks. Sections were floated at 45ºC ± 5ºC in a water bath (Lamb RA, model E/65) onto coated slides (Superfrost ® plus, BHD Laboratory Supplies, Poole, Dorset, UK) and stacked in a metal rack before being dried overnight in an oven at 55ºC (Lamb RA, model E28.5).

2.8. Tissue staining and Immunohistochemistry

2.8.1. Immunohistochemistry for paraffin embedded tissue

Slides were then dewaxed in xylene (5 min) and hydrated through graded alcohols at 20 second intervals (absolute alcohol; absolute alcohol; 95% alcohol and 70% alcohol) and washed in water.

2.8.2. Heat mediated antigen retrieval

Antigen retrieval was carried out to recover epitopes that undergo changes during the fixation process. Cross-linking of various unrelated proteins occurs and may result in a loss of immunoreactivity of the target antigen (Boenisch et al., 2001). Antigen retrieval was achieved by pressure cooking in 0.01M Citrate buffer (pH 6.0), under pressure for 5 minutes followed by a 20 minute cooling period before being placed in water.

2.8.3. Blocking non-specific sites

Endogenous peroxidase activity, which would otherwise interfere with horseradish peroxidase amplification, was blocked with 3% (v/v) hydrogen peroxide in methanol (both BDH laboratory supplies) for 30 min at room temperature. Slides were rinsed in tap water followed by 2 x 5 minute washes in Tris-buffered saline (TBS; 0.05M Tris-HCl, pH 7.4, 0.85% NaCl). The slides were removed individually from the TBS wash and dried carefully around the tissue section using tissue paper

This step was followed by an Avidin-Biotin block carried out according to the manufacturer's protocol (Vector Laboratories, Peterborough, UK) which prevents non specific endogenous avidin/biotin signalling. Avidin was added directly to the tissue section for an incubation period of 20 minutes, followed by 2 x 5 minute washes in TBS then incubated for a further 20 minutes with the Biotin.

Non-specific binding of the secondary antibody was prevented by incubation with the appropriate non-immune block. This consisted of a solution containing serum from the species in which the secondary antibody was raised diluted 1:4 in TBS containing 5% bovine serum albumin (BSA; Sigma). Blocking serum was added to each section for 30 minutes during which slides were kept in a humidified chamber at room temperature.

2.8.4. Primary antibodies

The blocking buffer was removed by tapping the slides on tissue paper and replaced with the primary antibody diluted in the appropriate blocking serum. The slides were

incubated overnight in a humidified chamber at 4ºC. Antibodies and the conditions used for each are shown in Table 2.9. Negative controls were included in each run where the non-immune block was left on slides and no primary antibody was added. The slides were subsequently washed in TBS (2 x 5 min).

Table 2.9: Primary antibodies used in immunohistochemisrty

Antibody	Species	Source	Dilution	Retrieval
DAZL	Mouse	Setotec	1:100	Citrate
PCNA	Rabbit	Novocastra	1:1000	Citrate
BrdU	Mouse	Roche Diagnostics	1:50	Citrate
Pdcd4	Goat	Santa Cruz	1: 100	Citrate
Cleaved caspase 3	Rabbit	Cell signalling	1:300	Citrate

2.8.5. Secondary antibodies

After the TBS washes, secondary biotinylated antibodies diluted 1:500 in the appropriate blocking serum were added to the sections and incubated for 30 minutes at room temperature (Table 2.10). The secondary antibodies used were raised against the host species of the primary antibodies.

Table 2.10: Summary of secondary antibodies used

Host species	Target species	Source	Dilution
Goat biotynalated	Rabbit	Dako	1:500
Goat	Mouse	Dako	1:500
Rabbit	Goat	Dako	1:500

2.8.6. Antigen detection and counterstaining

Following washes in TBS (2x5 minutes), the biotinylated secondary antibody was linked to horseradish peroxidase (HRP) by a 30 minute incubation with avidin-biotin-HRP complex (ABC-HRP; DAKO, High Wycombe, UK) diluted in Tris-HCl (pH 7.4) 1:1000. This was followed by two 5 minute washes in TBS.

2.8.7. DAB immunostaining

Antibody localisation was determined by application of liquid diaminobenzidine (DAB) substrate chromogen system (DAKO, High Wycombe, UK) for 1-5 min until

the brown positive staining in control sections was optimal; the colour reaction was stopped by immersion in water. This brown staining is due to DAB being an electron donor that oxidises in the presence of peroxidase in order for a colour change to take place. The successive stages of the immunohistochemical technique are summarised in Figure 2.10:

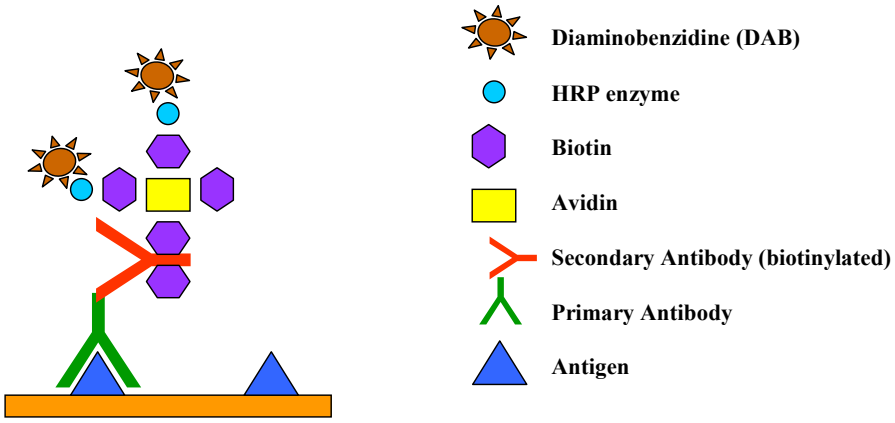

Figure 2.10: Principles of direct immunostaining showing the interaction between the biotynalated secondary antibody and the detection reaction using avidin-biotin HRP and DAB.

Harris's haematoxylin (Section 2.12) was used to counterstain the tissue section, staining the cell nuclei blue. Scotts tap (Section 2.12) water was used to develop the blue colour. Slides were rinsed in tap water and subsequently dehydrated in graded alcohols and cleared in two 5 min washes of xylene. Slides were then mounted with glass coverslips (VWR) using pertex (Cell Path). Immunostained sections were photographed using a Provis AX70 microscope (Olympus Optical, London, UK) fitted with a digital camera (Canon DS6031; Canon Europe, Amsterdam). Captured images were then transferred to a PC and compiled using Adobe Photoshop 7.0 (Adobe Systems Inc, CA, USA).

2.9. Radioimmunoassay

2.9.1. LH and FSH

All blood sampling and radioimmunoassay were performed by Judy McNeilly. After CO_2 administration, blood was collected by cardiac puncture using a heparinized syringe from mice before cervical dislocation. Blood samples were centrifuged at 8000rpm for 10 minutes for plasma separation and the plasma stored at -20C until required for assay assessment. The concentrations of plasma LH and FSH were measured by RIA using reagents supplied by the NIDDK with all samples for each hormone assayed repeated in duplicate. The reference preparations used were rat LH RP-1 and rat FSH RP-3, and the minimum detectable concentrations were 0.2 and 1.2 ng/ml for LH and FSH, respectively.

2.10. BrdU injections dose

Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is a synthetic analogue of thymidine commonly used in the detection of proliferating cells in living tissues. BrdU is incorporated into the newly synthesized DNA of replicating cells (during the S phase of the cell cycle), substituting thymidine during DNA replication. Cells which have been undergoing proliferating can then be detected immunohistochemically (Section 2.8) using an antibody specific for BrdU. Since BrdU can replace thymidine during DNA replication mutations can occur, therefore termination of the animals after injection is essential as there is a potential health hazard, although this is a rare event. BrdU was administered at a dose of 1mg/1ml=100ul via intraperitoneal injection physiological saline (0.9% NaCl, w/v).

2.11. Statistical analysis

Values are expressed as means ± SEM, and data were analysed using Student's unpaired *t*-test or one-way ANOVA following Bonferroni post test using GraphPad Prism (version 5, GraphPad software Inc., San Diego, CA). Chi-square (two-tailed) test was used to analyses data for survival vs. burst rate of follicles. Only when there

were statistically significant differences are these indicated by asterisks, * P<0.05, **P<0.01 and ***P<0.001.

2.12. Commonly used solutions

All chemicals were supplied by Sigma unless otherwise stated.

1% electropheresis gel:

1g agarose
45ml H2O
(Microwave 5 minutes med/high)
5ml TBE
0.5µl safeview

Acid alcohol: 70% ethanol
1% concentrated HCl

Bounin's solution: Picric acid can now only be bought by industry so Bounin's solution is made by Triangle Biomedical Sciences Ltd, Lancashire, UK.

4% NBF:

100ml	40% formaldehyde (BDH)
900ml	Distilled Water
4g	Sodium dihydrogen phosphate monohydrate (BDH)
6.5g	Disodium hydrogen phosphate anhydrous (BDH)

Citrate buffer: Citric acid 42.02g (monohydrate; Sigma)
Distilled H_2O 1900ml
Add concentrated NaOH to pH 5.5
Make up to 2L and pH to 6
Use as 0.01M, diluting in distilled H_2O

Harris's Haematoxylin:

2.5g Haematoxylin (Triangle Biomedical Sciences)
25ml absolute alcohol
50g aluminium potassium sulphate + 500ml distilled H_2O
Combine both solutions and boil
Add 1.25g Mercury oxide
Cool solution in ice then filter
Add 4ml glacial acetic acid/100ml Haematoxylin

Scott's Tap Water: Potassium chloride 10g
Magnesium sulphate 100g
Tap water 5L

TBS: Tris (Sigma) 60.5g
NaCl (Sigma) 87.6g
HCL (BDH) 300ml
Adjust to pH 7.4 using concentrated HCL

RIPA: (5X RIPA buffer)
ml 5M NaCl
25ml 1M Tris-HCl (pH 7.4)
5ml 0.5M EDTA
5g deoxycholate sodium
0.5g SDS
Protease inhibitor cocktail (Roche) was added to 1X RIPA at 100µl/ml.

SDS Gel Loading Buffer (Laemmli buffer):

62.5 mM Tris, pH6.8
2% SDS
1% B-mercaptoethanol
5-10% Glycerol

0.01% Bromophenol Blue

4% Stacking Gel: 0.75ml Acrylamide 40%

0.5ml 2% bis

1.25ml 1.25M Tris pH 6.8

0.1ml SDS 10%

7.65ml H_20

Polymerize with 100µl 10% APS and 25µl of Temed

10% Running Gel: 2.5ml Acrylamide 40%

0.5ml 2% bis

1ml 3.15M Tris, pH 8.8

0.1ml SDS 10%

4.42ml H20

Polymerize with 50µl 10% APS and 5µl of Temed

Electrophoresis buffer 10x (4°C):

192ml 1M Glycine

25ml 1M Tris base

10ml SDS 10%

Make up to 1000ml using dH_2O

Transfer buffer 1 (4°C):

192ml 1M Glycine

25ml 1M Tris base

10ml SDS 10%

200ml MeOH

Make up to 1000ml using dH_2O

Chapter 6: Ovarian role of putative DAZL target PDCD4

6.1. Introduction

The methods used in Chapter 5, whereby the DAZL consensus sequence (Venables et al., 2001) was employed to identify DAZL targets within the mouse genome had been previously exploited to identify potential DAZL candidates within the human genome (Ismail, MSc 2007). Once again a high number of potential targets were identified and by process of elimination by ovarian specificity and potential function, the candidate gene identified and selected for further investigation was programmed cell death factor 4 (PDCD4). PDCD4 was not originally identified in the mouse bioinformatics trawl, but on investigation into the expression of the consensus sequence within the mouse gene homologue it was revealed that a DAZL binding motif was located at the 3'mRNA of this potential target (Figure 6.1). Furthermore, with the understanding that DAZL affects litter size by a mechanism unknown, a potential function for PDCD4 in programmed cell death within the developing follicle resulting in an increase in ovulation and decrease in atresia in the Het females led to further investigation of this potential target.

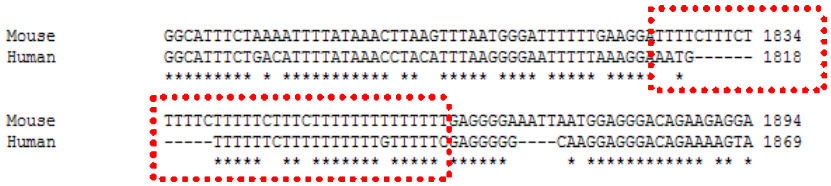

Figure 6.1: Human and mouse PDCD4 mRNA sequences exposing potential DAZL binding motifs present within the 3' UTR.

PDCD4 is also known as MA3 (Shibahara et al., 1995), TIS (Onishi et al., 1998) 197/15a (Azzoni et al., 1998) H731 (Cmarik et al., 1999) and DUG (Goke et al., 2002) and was originally isolated as a gene activated in cells during apoptosis and commonly up-regulated upon induction of cell death (Shibahara et al., 1995). Furthermore, PDCD4 has been identified as a translation factor which to bind to

eIF4A (Goke et al., 2002; Yang et al., 2003; 2004), inhibiting protein translation and expression is strongly associated with a decline in tumour progression. Latterly, PDCD4 has been briefly reported to be expressed abundantly in oocytes and in embryos through the two-cell stage (Jurisicova et al., 1998), but how and when it functions within the steroidogenically active ovary is unknown. In addition it has been reported that PDCD4 deficient mice which express many detrimental phenotypic characteristics (spontaneous lymphomas, significantly reduced life span, altered oncogenesis and inflammation) also develop multi-organ cysts which involved the ovary in 50% of animals (Hilliard et al., 2006). Furthermore, PDCD4 expression has been shown to be down-regulated in ovarian tumors (Bonome et al., 2005). Interestingly, unilateral ovarian hyperplasia and cystic tumour formation are also characteristic features of the DAZL KO ovary (McNeilly et al., SRF 2008) which might suggest that the lack of DAZL may be linked to a decrease in expression of PDCD4 controlled apoptotic control hence tumour growth. Thus, PDCD4 may be involved as a positive regulator in programmed cell death within the ovary or indeed as a functional component of the transcription mechanism involving DAZL and eIF4A. These potential connections were the major motivating factors for the studies in this chapter and in order to investigate the possibility that PDCD4 plays an active role associated with DAZL and in particular regulating programmed cell death, it was necessary to expand and clarify the understanding on PDCD4 expression and localisation within the ovary. These parameters were therefore investigated first and related to potential function, again recognising that the functional copy number of DAZL in the Het phenotype may be having interacting effects.

During these investigations of the potential role of PDCD4 as a DAZL target it became apparent that this cell death associated protein was abundantly expressed within the developing ovary, and of particular interest, was the difference in nuclear to cytoplasmic localisation within the corpus luteum. Further to these findings, induced follicle atresia, natural apoptosis associated with pro-oestrous, and interruption to pregnancy through the use of luteolytic mediators bromocriptine and cloprostenol were also investigated to determine potential functional roles of PDCD4

within the ovary. Having an understanding of the cellular and molecular mechanisms that activate and execute programmed cell death in the female germ line has implications for therapeutic management as does understanding the importance of DAZL expression within oocyte development.

6.2. Materials and Methods

6.2.1. Identification of PDCD4

In brief, by the same *in silico* approached used in Chapter 5 (Section 5.2) the human genome was subjected to a BLAST search (NB: the initial BLAST search was performed by Dr Wail Ismail with all subsequent analysis and investigations performed by myself) using the different permutations of the DAZL binding motif (Venables et al., 2001) to identify potential putative RNA targets to which DAZL may bind and regulate mRNA expression. As mentioned a potential DAZL binding site is located in the murine PDCD4 sequence, however investigation of the patterns of expression and elucidation of potential function were required. To verify PDCD4 expression within Wt and Het oocytes (d21) and Wt and Het ovarian tissues (d21 and d10 mice) specific oligonucleotide primer sets were designed for mouse PDCD4 and used to detect reverse transcription products by conventional PCR (Section 2.3.4). In addition real-time light cycler SYBR green PCR was performed to detect differences in PDCD4 transcript number between the two genotypes (Section 2.4). The protein expression of PDCD4 was detected using immunohistochemistry, performed using the standard protocol (Section 2.8).

6.2.2. Role of PDCD4 in ovarian apoptosis

To investigate the potential roles of PDCD4 as a putative DAZL target the genes, in additions to its recognised pro-apoptotic function, studies were designed using mice to observe natural atresia in follicles and corpora lutea, induced follicle atresia, and disrupted CL function through induced luteolysis.

6.2.2.1.Natural ovarian apoptosis

Pro-oestrus animals were used to investigate natural atresia associated with corpus luteum degeneration during the oestrous cycle, whereby old luteinized corpora lutea undergoing functional regression should be present. Cycling adult virgin mice were smeared daily (Judy McNeilly) to determine the stage of the oestrous cycle. The sample vaginal smears were analysed under a microscope to determine the cytology of the cells present, hence determine cycle stage. Animals were selected depending on the stage of cycle for ovarian collection and further analysis.

6.2.2.2.Induced apoptosis: modulation by FSH withdrawal

In order to investigate apoptosis within developing follicles a regime was designed whereby folliculogenesis was induced by the administration of recombinant human FSH (rech FSH) to pre-pubertal mice (d21) followed by FSH withdrawal. The withdrawal of FSH from the system was achieved by neutralising the exogenous rech FSH by treatment with a rabbit anti-human FSH antibody (M91: 100µl ip) and endogenous FSH secretion was also inhibited by treatment with ovine follicular fluid (oFF; 100µl ip: Crawford et al., 2004) as a source of inhibin (Figure 6.2). The antibody against FSH should immuno-neutralise the circulating recombinant FSH whereas the oFF inhibits secretion of any endogenous FSH present through a direct action on the pituitary (Brown et al., 2001). This regime of FSH stimulation followed by acute withdrawal was hypothesised to induce follicle atresia. Four animals were used in each treatment group.

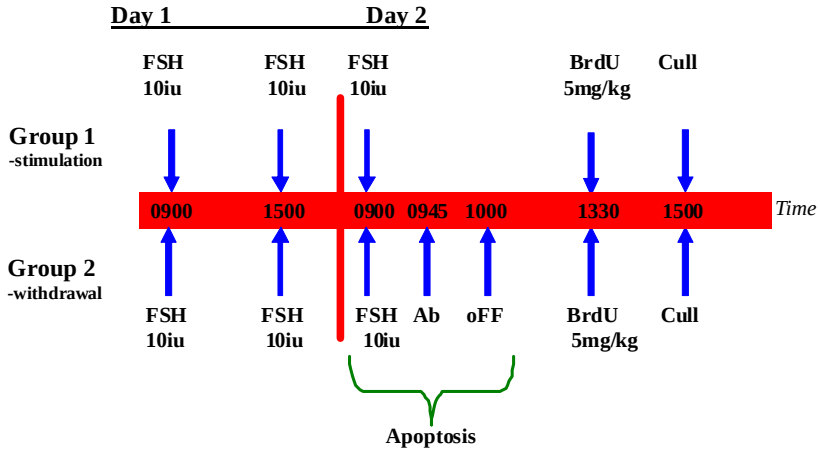

Figure 6.2: Schematic summary of FSH treatment regime applied to d21 pre-pubertal mice to induced folliculogenesis (Group 1; n=4) and induced apoptosis (Group 2; n=4) by treatment and withdrawal of FSH.

6.2.2.3.Chemically induced luteolysis

Luteolysis is the natural structural and functional degradation of the CL. However it can be chemically induced by direct and indirect methods. Bromocriptine and cloprostenol were selected as luteolytic agents to investigate cell death within the CL and in understanding their disruptive mechanism to induce cell death it may aid the identification of potential roles of PDCD4 protein within the ovary. Bromocriptine is a chemical dopamine agonist which inhibits prolactin synthesis and secretion by directly binding to dopamine D2 receptors on the pituitary lactotrophs (Vance et al., 1984). A decrease in prolactin results in a reduction of progesterone and hence induces luteolysis within the CL of the pregnant rodent (Zetser et al., 2001). Cloprostenol is a functional analogue of prostaglandin F2α having a specific luteolytic action at the ovarian level. It causes functional and morphological regression of the CL in ungulates, marmosets and rodents (Nancarrow et al., 1982; Summers et al., 1985; Torjesen and Aakvaag, 1986) followed by a return to oestrous and normal ovulation. The functional hypothalamic-pituitary-ovarian axis and the disruptions caused by bromocriptine and cloprostenol are summarised in Figure 6.3.

Female mice caged with a stud male were checked daily for the appearance of vaginal plugs indicating mating and potential conception. On the seventh day of gestation the females were injected intra peritoneal (ip) with bromocriptine (4mg/kg; 120μg/30g mouse, Sigma) (2-bromo-α-ergocrytine.methanesulfonate salt B2134-25MG 095K1528) or with cloprosetenol ((Estrumate synthetic Prostaglandin Schering-Plough Animal Health) dose 5μg/mouse)). Sixteen hours after the luteolytic treatments, the animals were given injections of BrdU, (5mg/kg ip) and subsequently the mice were euthanized after an additional two hours. Body weight was recorded, blood was collected by cardiac puncture and the ovaries were removed and weighed. The number of visible corpora lutea were counted, the condition of the uterus noted and the number of implantation sites were recorded. Ovaries were fixed in NBF (Section 2.7), for subsequent analysis and blood collected for LH and FSH radioimmunoassay (Section 2.9, performed by Judy McNeilly).

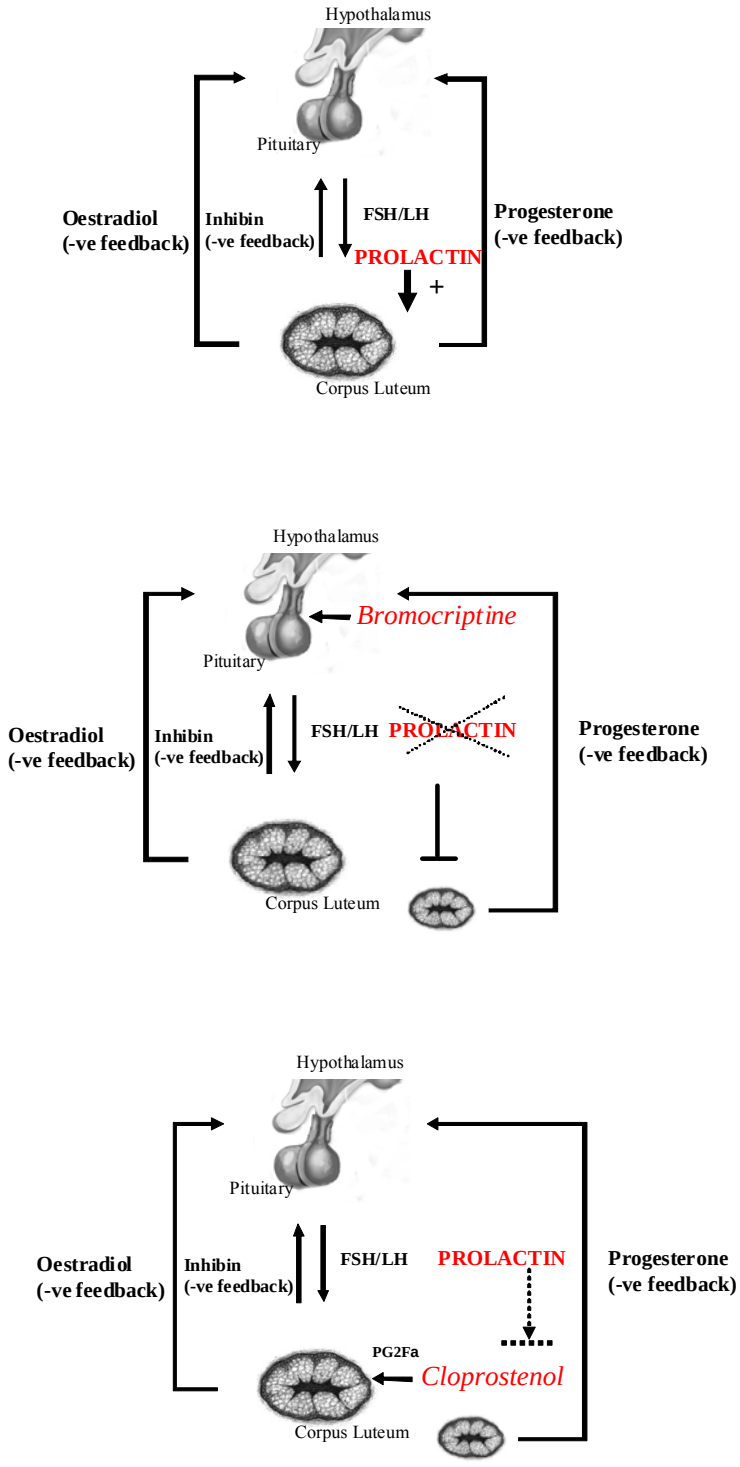

Figure 6.3: Summary of hypothalamic-pituitary-ovarian interaction during the luteal phase: actions of bromocriptine and cloprostenol on prolactin function and luteolysis.

6.2.3. Analysis

To assess the degree of induced atresia, whether natural, by FSH withdrawal or by inducement of luteolysis, ovaries from the different treatment groups were sectioned and immunostained for markers of proliferation (PCNA or BrdU (Section 2.8)) and the apoptotic marker cleaved caspase 3, in addition to PDCD4, the protein of interest. Observations were made on the relationship between PDCD4 localisation and expression compared to that of cleaved caspase 3 expression. Visual analysis of cleaved caspase 3 expression was performed from the ovaries which had been subjected to FSH stimulation/withdrawal. In addition mRNA expression of PDCD4 and cleaved caspase 3 was analysed using SYBR green RT-PCR (Section 2.4.2). The number of corpora luteum were recorded in the induced luteloysis groups in addition to the appearance of the nuclear compared to cytoplasmic localised expression of PDCD4 in comparison to the pregnant controls.

6.3. Results

6.3.1. PDCD4 as a potential DAZL target

It is assumed that the functional copy number of DAZL would potentially determine the transcription levels of functional DAZL targets. Therefore the expression of the recently identified putative target PDCD4 was firstly compared between the DAZL Wt and Het females. PDCD4 mRNA expression levels were investigated in d21 oocyte pools (localised DAZL expression) and d10 ovaries (uniform population of same stage oocytes) as previously used in Chapter 3. The expression levels of PDCD4 were unchanged between the Wt and Het samples at both d21 (oocyte pools) and d10 (ovaries) (Figure 6.4).

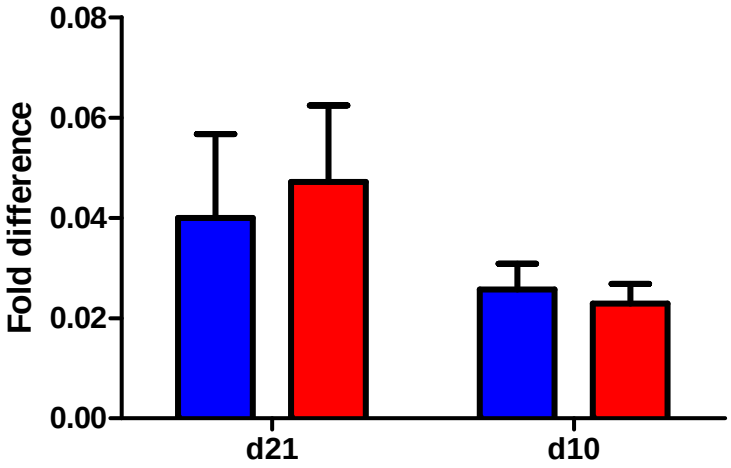

Figure 6.4: Quantitative analysis of PDCD4 mRNA fold difference between Wt and Het in d21 oocyte samples (n=5) and d10 ovaries (n=5). Values are means ± SEM (Wt and Het).

6.3.2. Novel expression of PDCD4 in the mouse ovary

Having confirmed that PDCD4 mRNA was present in the mouse ovary, and although no differences in mRNA expression were detected between the Wt and Het, we then established the pattern of PDCD4 protein expression within ovarian tissue using immunohistochemistry. Initially d21 ovary, adult ovary with corpus luteum present, oviduct, DAZL KO d21 ovary and tumor samples from a DAZL KO ovary were analysed to enable a more informed interpretation of PDCD4 expression within the female reproductive tract. Review of PDCD4 protein immunoexpression revealed positive cytoplasmic localisation within the oocytes of the d21 ovary, both in the

small primordial oocytes and oocytes within the larger pre/antral follicles (Figure 6.5 A). In addition PDCD4 was located to the granulosa cell cytoplasm, with light immunostaining present in the surrounding thecal layers (Figure 6.5 A). PDCD4 protein expression was additionally localised to the ovarian surface epithelium (OSE) surrounding the ovary where expression was strong (Figure 6.5 B) and to the steroidogenic cell of the corpus luteum (Figure 6.5 B). Within the corpus luteum PDCD4 was expressed in both the cytoplasm and in the nucleus, which prompted further investigation (Section 6.3.4 and 6.3.5). Furthermore, the oviduct epithelial cells intensely expressed PDCD4 protein (Figure 6.5 C) as did the OSE and cells within the KO ovary (Figure 6.5 D). In the KO ovarian tumor and consistent with previous reports illuminating the decrease in PDCD4 expression associated with tumor progression, PDCD4 immunoexpression appeared reduced in the ovarian tumor sample (Figure 6.5 E).

The observations of PDCD4 expression and the striking nuclear localization in cells within the CL compared to cytoplasmic expression in the granulosa cells of follicles suggested possible translocation of this protein with regards to its active function. These observations subsequently contributed to the series of investigations addressing natural and induced apoptotic functions within the ovary to identify potential function of PDCD4 as a pro-apoptotic factor.

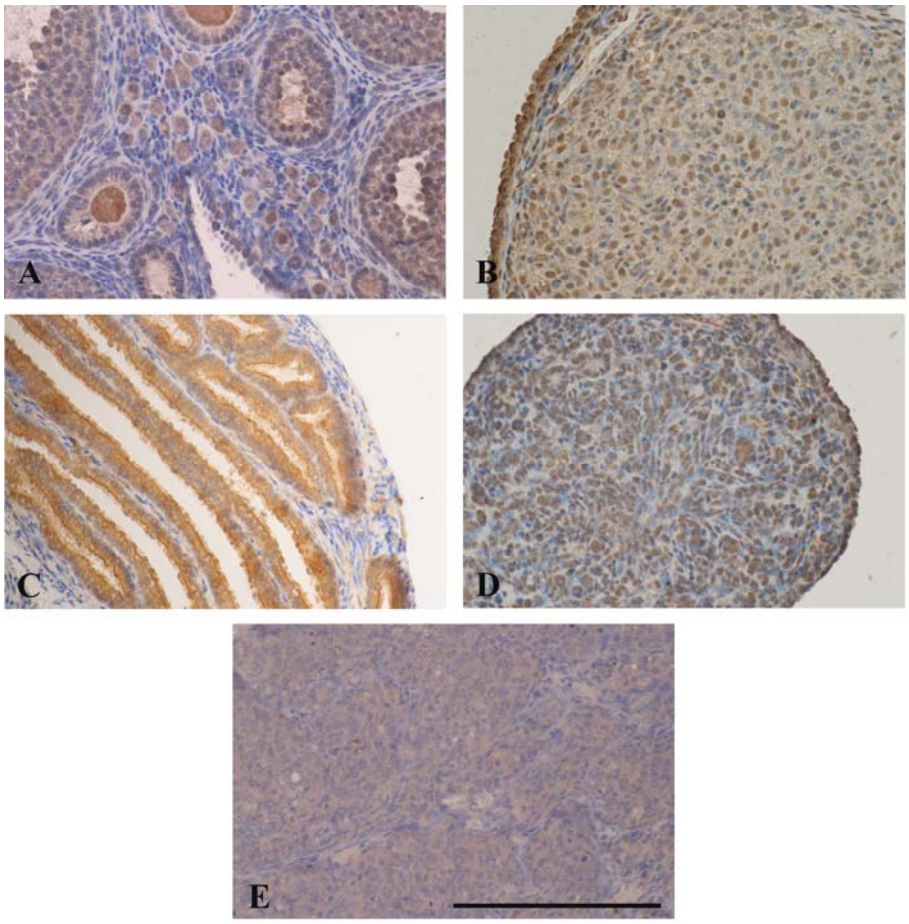

Figure 6.5: Immunoexpression of PCDC4 in representative sections of d21 ovary (A), adult ovary corpus luteum (B), oviduct (C), d21 DAZL KO ovary (D) and a tumor from a DAZL KO ovary. Scale bar represents 100μm.

6.3.3. PDCD4 mRNA and protein expression during follicle apoptosis

Evaluation of cell proliferation markers, PCNA and BrdU were investigated using immunohistochemistry after FSH stimulation or after FSH withdrawal of d21 ovaries, to enable the detection of proliferating cells (Figure 6.6). PCNA is a cell cycle protein which holds DNA polymerase delta to DNA with expression increasing during the G1-phase, peaking at the S-phase, and declining during the G2/M-phases of the cell cycle (Foley et al., 1993). PCNA was detected within granulosa cells and stromal cells (Figure 6.6 A and B) of the two treatment groups. As a result of the prolonged expression through the cell cycle no obvious differences were observed

between the two treatment groups, although cells did show different degrees of staining intensity which one would expect. Furthermore, BrdU is present only the S phase of the cell cycle within which it is incorporated into newly synthesized DNA of replicating cells, substituting thymidine during replication. In comparing the subsequent sections (Figure 6.6 C and 6.6 D), large atretic follicles have a substantial reduction in BrdU protein expression compared to the surrounding proliferating follicles.

In comparison to these intrinsic proliferative markers, cleaved caspase 3 was used to identify apoptotic regions within the ovary and used as a comparative marker for the expression of the pro-apoptotic PDCD4. An increase in cleaved caspase 3 expression was notably observed after the FSH withdrawal treatment, clearly indicating follicle atresia (Figure 6.6 E). Both an increase in pyknotic nuclei and an increase in cytoplasmic cell degradation was observed. This increased cleaved caspase 3 expression was not observed in the FSH stimulation group (Figure 6.6 F). Interestingly even in the classified atretic follicles identified by the expression of cleaved caspase 3 and pyknotic nuclei, cell proliferation was also observed with reference to BrdU and PCNA positive immunoexpression, although expression of BrdU was reduced. There was no identifiable difference in the localization or intensity of PDCD4 protein between the two FSH treatment groups. If PDCD4 is acting as a pro-apoptotic factor in follicles one would presume either a decrease in expression at the onset of induced follicle atresia or a functional translocation to activate its activity within the apoptotic cell of the follicle.

In order to further investigate the possibility that PDCD4 may play a role in ovarian apoptosis, ovaries from the d21 animals subjected to the FSH stimulation and withdrawal were additionally used to analyse cleaved caspase 3 and PDCD4 mRNA expression. Interestingly the expression of cleaved caspase 3 was reduced after the FSH withdrawal treatment compared to FSH stimulation treatment, in contrast to the apparent increase in protein expression. However, cleaved caspase 3 mRNA may have become degraded as a result of apoptosis despite protein remaining. In support

of the absence of change in the expression of PDCD4 protein, no difference in PDCD4 mRNA expression was observed between the two treatment groups (Figure 6.7), further suggesting that PDCD4 is not involved in induced follicle atresia regardless of its association as a pro-apoptotic factor.

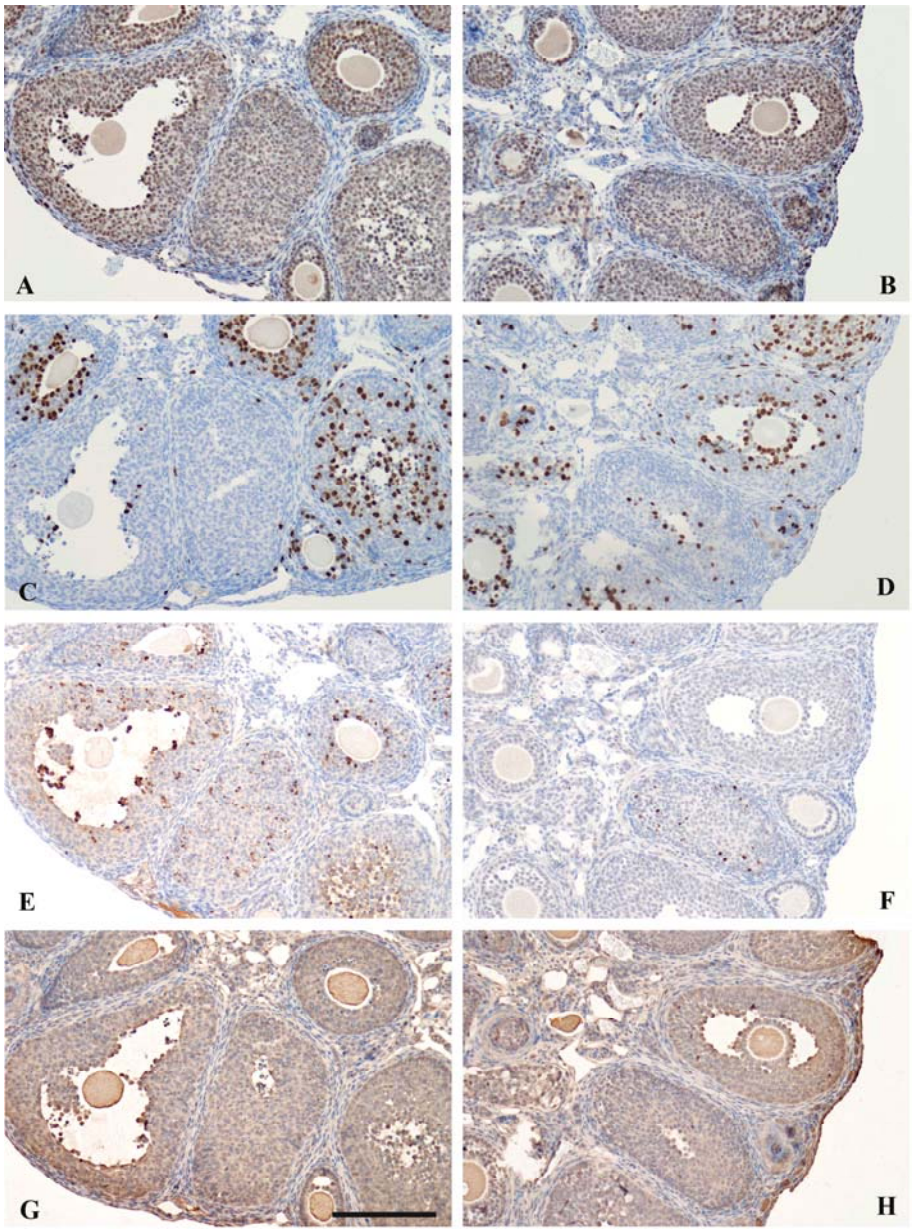

Figure 6.6: Immunoexpression of PCNA (A: FSH withdrawal and B: FSH stimulation), BrdU (C: FSH withdrawal and D: FSH stimulation), Cleaved caspase 3 (E: FSH withdrawal and F: FSH stimulation) and PCDC4 (G: FSH withdrawal and H: FSH stimulation). Scale bar represents 100µm.

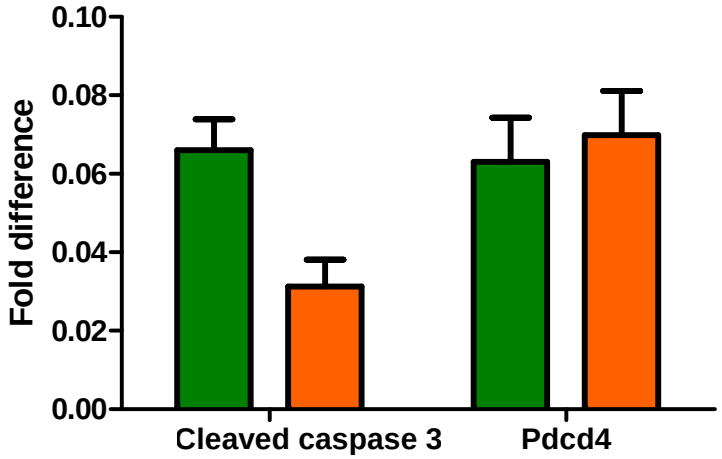

Figure 6.7: Quantitative analysis of cleaved caspase 3 and PDCD4 mRNA levels after FSH stimulated compared to FSH withdrawal treatment d21 ovaries (n=4). Values are means ± SEM.

6.3.4. PDCD4 protein expression during natural apoptosis

In order to investigate a process of natural apoptosis within the ovary and in addition to the PDCD4 protein localisation identified within the CL, pro-estrous animals were screened again for the two proliferative markers PCNA and BrdU (Figure 6.8 A-D). Representative images from two different animals confirmed that there is extensive proliferation occurring in the pro-estrous ovary, with a reduced expression of PCNA within the corpora luteum and only a few BrdU positive cells. Apoptotic follicles were observed within the pro-estrous ovaries, and as indicated by cleaved caspase 3 protein expression, very few cells within the CL are apoptotic (Figure 6.8 E-F). Furthermore, the expression of PDCD4 notably changed within the CLs examined, ranging from light cytoplasmic expression with limited nuclear expression (Figure 6.8 G) to more intensely expression and localised to the nucleus in the steroidogenic cells (Figure 6.8 H). These differences observed in CL PDCD4 immunoexpression, visually correlated to the expression of cleaved caspase 3, whereby, when PDCD4 was light with reduced nuclear expression a higher number of positive cleaved caspase 3 cells were present with the CL. Conversely, when nuclear PDCD4 expression was observed in the CL, little or no cleaved caspase 3 was identified.

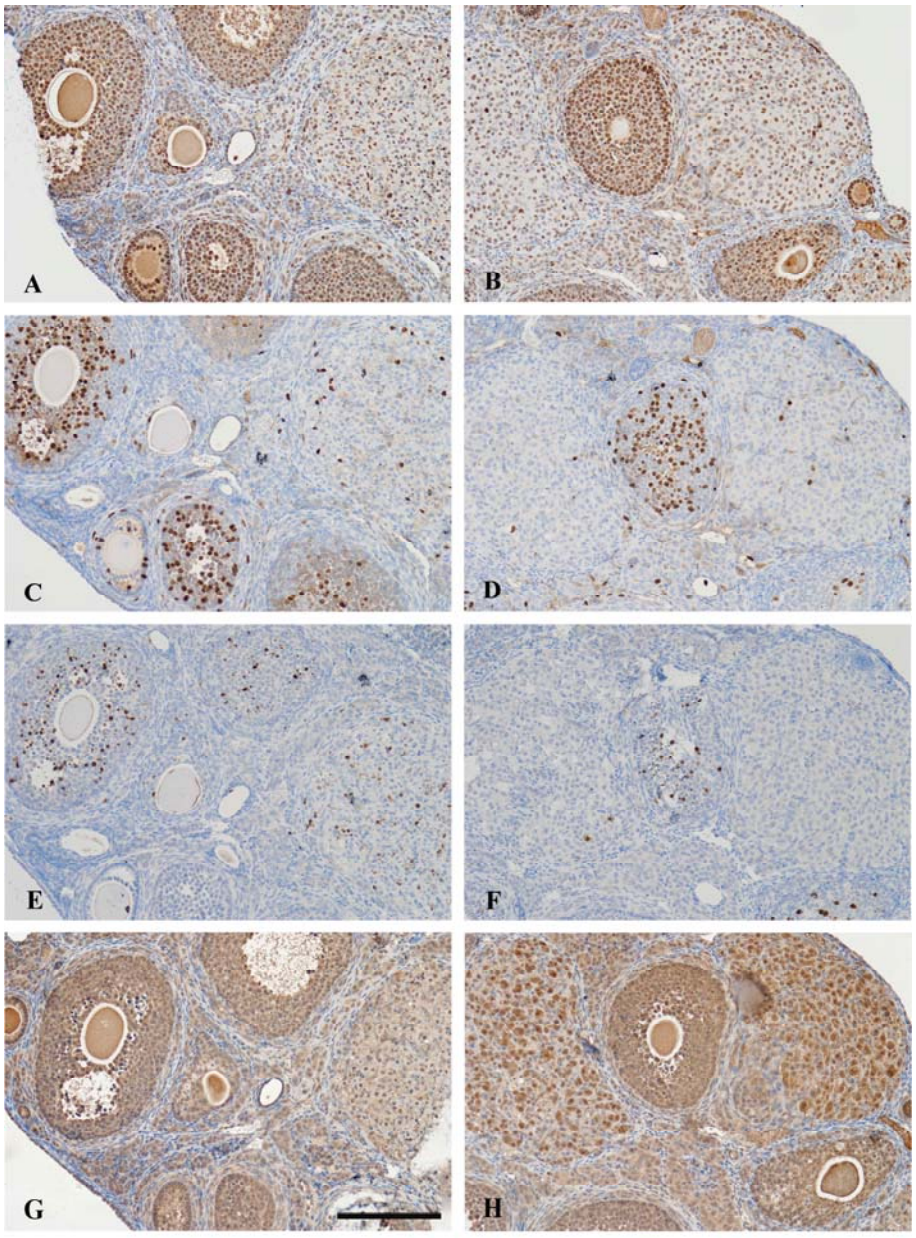

Figure 6.8: Representative photomicrographs depicting immunoexpression of proliferation markers PCNA (A and B), BrdU (C and D), apoptotic marker Cleaved caspase 3 (E and F) and protein under investigation PCDC4 (G and H) in pro-estrous animals. Scale bar represents 100µm.

6.3.5. PDCD4 protein expression in chemically induced luteolysis

Exposure to bromocriptine and cloprostenol induced functional luteolysis and disruption of pregnancy compared to the non-treated pregnant controls. Variation in the effects on the uterus, implantation sites and ovary were recorded (Table 6.1).

Table 6.1: Ovarian and uterine observations of chemically induced luteolysis.

Treatment	Day of pregnancy	Observation
Bromocriptine	8	Uterus red and swollen
	8	Very swollen
	9	Swollen enlarged uterus
	8	8 visible implantation sites
Cloprostenol	6	10 visible implantation sites
	6	6 visible implantation sites
	7	11 visible implantation sites
	8	White ovaries, implantations but not healthy
	9	White ovaries, implantations but not healthy
Control	8	11 visible implantation sites
	7	9 visible implantation sites
	8	12 visible implantation sites

Changes in CLs were clearly visible in the different treatment groups when compared to the non-treated control, with the result that abnormally unorganized areas of cells were present. The structural analysis of the CL suggest that upon chemically-induced luteolyis, CL degradation and structural regression is accompanied with an infiltration of epithelial type cells, which do not express PDCD4 protein (Figure 6.9 C, D and F). The degree of abnormally organized cells and infiltration of epithelial type cells varied substantially. It was clearly evident that the CL present in the non-treated pregnant controls (Figure 6.8 G and H) were more uniform in structure presenting with healthy round steroidogenic cells in contrast to the treated groups showing luteolyic changes in the CLs. In addition the control animal showed a more overall cytoplasmic expression of PDCD4 in comparison to PDCD4 expression being nuclear and cytoplasmic in the treated animals. Furthermore, overall expression of PDCD4 was higher in the treated animals, thus

suggesting that the intensity and nuclear localization within the CL is linked to the morphological changes and structural regression as a result of disruption of prolactin function. Although not shown there was very little cleaved caspase expression present in the CLs analysed from both the treatments inducing luteolysis, with the majoring of CLs remaining negative. The highest proportion of caspase 3 expressing CL was from the bromocriptine treatment groups, suggesting that the actions of the two luteolytic factor are possibly inducing regression at a different rate. Differences were also notable between the pregnant CLs and the pro-estrous CLs (Figure 6.9 A and B (pregnant) and G and H (pro-estrous)), with the pro-estrous CLs expressing a higher degree of nuclear expression compared to the mainly cytoplasmic expression of PDCD4 in the pregnant CLs.

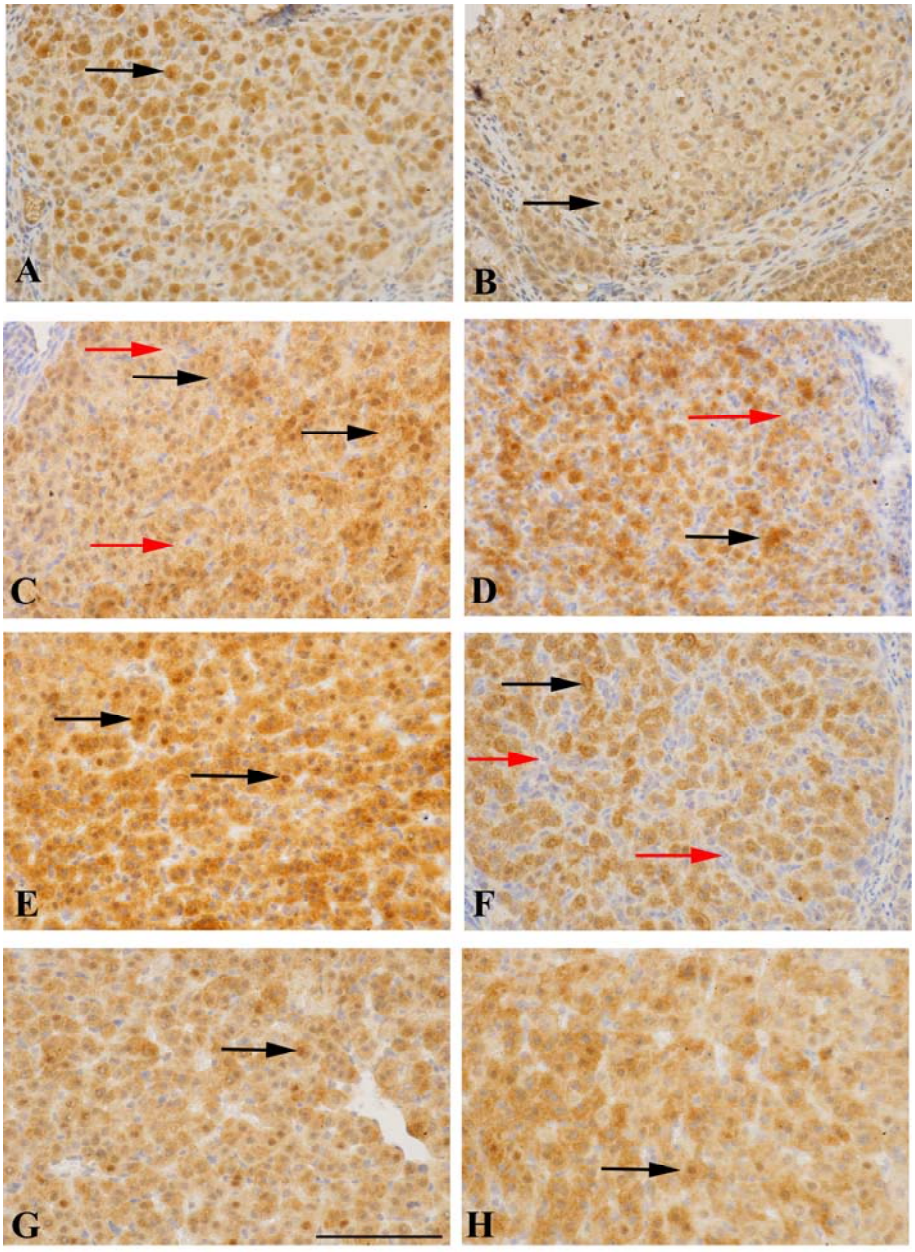

Figure 6.9: Representative photomicrographs depicting immunoexpression of PDCD4 in CLs of pro-estrous animals (A and B), bromocriptine (C and D), cloprostenol (E and F) and the non-treated pregnant control (G and H). Black arrows indicate representative areas with nuclear localization, red arrows indicate representative areas of epithelial cell infiltration. Scale bar represents 100µm.

The plasma levels of the gonadotrophins LH and FSH were measured after exposure to the luteolytic factor bromocriptine which disrupt prolactin secretion, and cloprostenol a PGF2α analogue which acts directly with the CL. FSH levels were comparable to in all treatment groups. However there was a reduction in LH after treatment with cloprostenol compared to LH levels observed in the control and bromocriptine animals, although due to the large variations observed in LH levels this reduction was not significant (Figure 6.10).

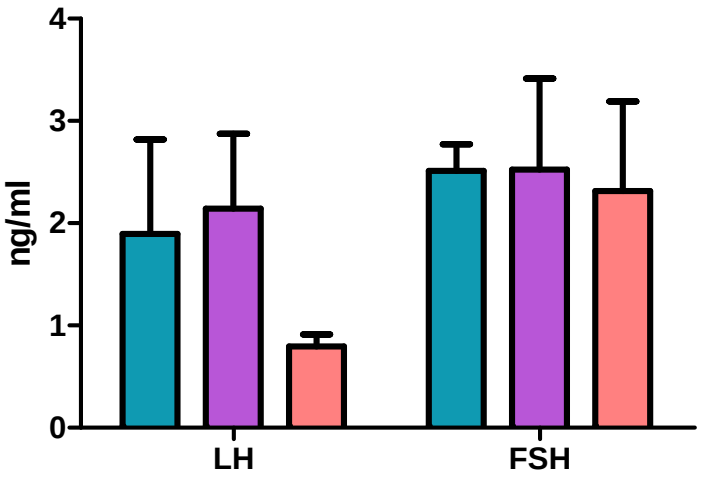

Figure 6.10: Changes in mean (±SEM) plasma concentrations of LH and FSH in non-treated pregnant controls and after luteolytic induction treatments bromocriptine and cloprostenol.

6.4. Discussion

6.4.1. PDCD4 as a putative DAZL target

This study is the first to identify PDCD4 not only as a potential DAZL mRNA target but additionally reports the temporal expression of PDCD4 within ovarian tissue. The initial identification of PDCD4 led to an extensive investigation in an attempt to elucidate probable PDCD4 functions within the ovary, with particular reference to the CL. The identification of the DAZL consensus sequence within the mouse PDCD4 homologue suggests a potential role for this gene in DAZL-mediated regulation and control. As previously investigated (Chapter 3 and 4), the functional copy number of DAZL is believed to be affecting the productivity of folliculogenesis and subsequently increasing the rate of ovulation and hence number of offspring in the Hets. Comparison of PDCD4 mRNA between Wt and Het mice, no differences were detectable between PDCD4 mRNA levels in d21 oocytes or d10 ovaries were observed, suggesting the control mechanisms of the mRNA expression are not altered as a result of reduced functional copy of DAZL. Although, it cannot be completely ruled out that PDCD4 is a functional target of DAZL until *in vivo* interactions are disproven. This will require further optimisation of the immunoprecipitation technique (Chapter 4) for ovarian tissue in addition to investigating different approaches altogether to determine *in vivo* interactions.

6.4.2. Potential ovarian function of PDCD4

There are limited publications describing the expression of PDCD4 within the ovary. It has been detected within the oocyte and throughout blasotcyst development in the mouse, and the study states that its function is unknown although associated with apoptosis (Jurisicova et al., 1998). Furthermore, from a microarray study comparing human ovarian tumours, PDCD4 mRNA expression was shown to be notably decreased. Again elucidation of its function was not discussed (Bonome et al., 2005). However, this decrease in expression may be due to cellular transformation and progression of tumour growth rather than representative of a role for PDCD4 in ovarian function. Therefore we report here for the first time the temporal expression of PDCD4 within the mouse ovary, in addition to suggesting potential involvement of this protein in functional luteolysis. Evidence has been presented that PDCD4 is

detected at the mRNA and protein level in oocytes, which is supported by previous findings (Jurisicova et al., 1998), and additionally, protein has been localised to granulosa cells, the ovarian surface epithelium, oviduct epithelium and luteinised granulosa cells. Due to this extensive expression of PDCD4 in the female reproductive tissue in addition to its host of roles in pro-apoptotic mechanisms (Shibahara et al., 1995), the likelihood of PDCD4 being a direct target of the oocyte specific DAZL remains low. However PDCD4 may well play a significant role in the control and development of both the oocytes and surrounding ovarian structures. This chapter has highlighted that there are still many mechanisms within the ovary which remain unsolved and with regards to intra/extracellular control of CL during luteolysis the striking nuclear expression of PDCD4 cannot be overlooked. To understand the possible ovarian function of PDCD4 an understanding in to its functional role elsewhere has been examined and discussed.

6.4.3. Function of PDCD4

PDCD4 was originally isolated in a search for genes that were activated as a result of apoptosis (Shibahara et al., 1995). In addition work investigating species homology indicates that PDCD4 is highly conserved during evolution indicating its importance in biological function (Lankat-Buttgereit et al., 2004) and has been related to a variety of functions within different tissue and cell systems but how it actually works is still to be fully elucidated. More recently, interests lies in the observation that PDCD4 has a tumor suppressor functioning by targeting and inhibiting translation and transformation. It has been identified that translation initiation factor (eIF4A) is a major binding component of PDCD4 (Yang et al., 2003) and like the translation initiation factor 4G (eIF4G), PDCD4 can interact with the RNA helicase eIF4A via its component MA3 domains. This competitive binding between PDCD4 and eIF4G results in an inhibition of translation by interfering with the assembly of the initiation complex consisting of the translation factors eIF4G, eIF4E and eIF4A (Goke et al., 2002; Yang et al., 2003). As a result, it seems that in the absence of PDCD4, eIF4A would be left unchecked to increase the amount of inefficiently translated mRNAs, thereby causing the cells to proliferate aberrantly leading to cancer (Jansen et al., 2005). It is important to note that this initiation complex has also been implicated in

the control of DAZL mediate transcription in Xenopus, whereby DAZL binds to Poly (A) binding proteins which are actively involved in the translation initiation complex (Gray et al., 2000; Collier et al., 2005).

Studies performed in PDCD4 deficient mice support the role of PDCD4 as a suppressor of tumorgenesis *in vivo* and additionally indicate its involvement in inflammation initiation by selectively inhibiting protein translation in the immune system (Hilliard et al., 2006). It has been shown in culture that the expression of PDCD4 is dramatically up-regulated in association with early event apoptosis as a result of serum starvation. Furthermore, it has been suggested that PDCD4 may be involved in both the intrinsic and extrinsic pathways of apoptosis (Goke et al., 2002).

Interestingly, unlike other documented translation factors that are constitutively expressed under physiological conditions, PDCD4 is normally expressed at low levels but is dramatically up-regulated under conditions that induce programmed cell death or transformation (Shibahara et al., 1995; Onishi et al., 1998; Goke et al., 2002). Therefore, the differences observed in PDCD4 immunoexpression at the time of functional luteolysis within the CL sequentially determine how much functional translation suppression is occurring regarding protein initiation and hence molecular function of PDCD4.

In the present study PDCD4 was predominately localised to the cytoplasm of granulosa cells of both healthy and atretic follicles and oocytes, with the notable difference in cellular compartmentalisation observed in the CL, most probably as a result of stage of regression and luteinisation. Thus, the differences observed further suggest that PDCD4 is an active molecular component within the CL, almost certainly associated with cell death. In light of these findings and in support of the difference observed, PDCD4 has been reported to be localised to the cytoplasm (Yang et al., 2003) and to the nucleus (Schlichter et al., 2001a; b). In a subsequent study PDCD4 protein was detected in the cytoplasm and/or in the nucleus, depending on the cells or their growth state (Yoshinaga et al., 1999). Collectively these

observations suggest that in addition to the reported roles of PDCD4 in protein translation within the cytoplasm, PDCD4 may have an important functional role within the nucleus. To clarify PDCD4 localisation, a further (Bohm et al., 2003) investigation revealed that PDCD4 can actually shuttle between the cytoplasm and nucleus, with nuclear location dominantly selected under normal growth conditions. In contrast to normal growth conditions, in serum depleted cells PDCD4 protein is located in the cytoplasm (Bohm et al., 2003). These findings suggest that this transfer in localisation from the cytoplasm to the nucleus is responsible for active translation inhibition thereby initiating apoptosis. In contradiction to this, it has been suggested that nuclear accumulation of PDCD4 occurs prior to apoptosis (Zhang et al., 2006). More recently it has been identified that phosphorylation of PCDC4 by Akt is the cause of the nuclear translocation (Palamarchuk et al., 2005) and phosphorylation of PDCD4 *in vitro* and *in vivo* occurs in a P13K-dependent manner, causing nuclear translocation. This action of phosphorlation inactivates PDCD4 in its function as an inhibitor of AP-1 mediated transcription. Members of the TGFβ superfamily have also been implicated in PDCD4 regulation, with PDCD4 increasing after treatment of transfected PDCD4 Huh7 cells with transforming growth factor-β. This increase in PDCD4 not only coincided with the occurrence of apoptosis and caspase activation but was reversed by the transfection of Smad7 (a known antagonist of TGFβ1) suggesting that Smad7 protect cells from TGF β1 mediated apoptosis (Zhang et al., 2006). Although somewhat under investigated, these studies suggest that PDCD4 may function in both the nucleus and the cytoplasm, with nuclear expression associated with normal growth and cytoplasmic expression associated with apoptosis. Furthermore, translocation from the cytoplasm to the nucleus may be associated with inactivation of PDCD4 and initiation of eIF4G transcription.

A coincidental common linkage has been formed between catenins and cadherins which have been identified both as putative DAZL targets as part of the bioinformatics trawl in Chapter 5, and to PDCD4. It has been shown that knockdown of PDCD4 expression stimulates the translocation of β-catenin into the nuclei, which in turn activates β-cateninsol T cell factor (Tcf)-dependent

transcription, and AP-1 dependent transcription. Thus, it has been suggested that PDCD4 regulates the expression of E-cadherin since both mRNA and protein levels of E-cadherin are decreased as a result of PDCD4 knock down. Additionally E-cadherin is a binding partner of β-catenin and therefore a decrease in E-cadherin expression resulted in an increase of cytoplasmic free β-catenin (Wang et al., 2007). Given that these two proteins, β-catenin and E-cadherin, are both potentially regulated by PDCD4 and catenin-1 and N-cadherin have been identified as putative targets of DAZL, communication between these groups of molecules may be linked and, although not by a directly functional interaction, may play important roles in translational control of interacting mechanisms.

More recently novel findings have identified that suppression of PDCD4 expression is vital for the invasive activity of COX-2 mediated by PGE (2) and IL-8, and that PDCD4 increases TIMP-2 expression to inhibit breast cancer cell invasion (Nieves-Alicea et al., 2008). COX-2, TIMPs and PGE are all recognised as regulatory factors within the CL and most certainly play a dominant role in luteolysis (Bardin, 1970; Smith et al., 1999; Wiltbank and Ottobre, 2003). It is well established that prostaglandins are involved in initiation of luteolysis in a number of species including rodents (Bardin, 1970; Smith et al., 1999; Wiltbank and Ottobre, 2003). With these data a plausible function for PDCD4 within the CL may be that increased expression associated with cytoplasmic localisation could increase TIMP 2 and hence aid structural regression of the CL. This suggestion is supported by studies performed in rats where the expression of TIMP-2 mRNA in the regressing CL suggests an involvement in luteal demise (Simpson et al., 2001).

The identification and molecular function of PDCD4 are recent discoveries and despite the documentation of potential roles the molecular function remains to be fully investigated, although some aspects of its function are beginning to emerge. The main functions remain to be associated with protein translation with PDCD4 and eIF4G having similar homolog thus being involved in translation suppression within the cytoplasmic compartment. This localisation is enhanced as a direct result of serum starvation, inducing apoptosis, with nuclear representation associated with

normal growth. All these documented mechanisms are transferable to the ovary, and in particular to the CL, where we observe an increase in cytoplasmic expression of PDCD4 as a result of removal of the luteolytic effects of prolactin. This increase in PDCD4 cytoplasmic intensity is accompanied by an increase in nuclear expression and further augments the possibilities of multiple functions within different cellular compartments. In addition to these findings it has also been shown that PDCD4 has intrinsic RNA binding properties although this has not been extensively investigated (Bohm et al., 2003). We can suggest that cytoplasmic expression is aiding the pro-apoptotic mechanisms of PDCD4 whereby protein translation is hindered allowing programmed cell death to occur. Furthermore nuclear expression is exacerbating the signalling outcomes and could be contributing indirectly to the cell fate. Even though this is the first study suggesting an active role of PDCD4 within the CL, the identity of RNA targets would additionally aid the clarification of the mechanistic properties of PDCD4. In conclusion these speculative suggestions require functional confirmation, with the need to amalgamate all the potential associated systems to collate an overall theory of PDCD4 function and apply them to the ovarian requirements.

6.4.4. PCNA v BrdU

PCNA and BrdU were utilised in the current studies as markers of cell proliferation, and as comparative factors for PDCD4 immunoexpression. In all studies, a large proportion of cells were actively labelled for PCNA compared to the BrdU. It has been recognized that PCNA can be continuously expressed in cells that are not actively dividing possibly due to its long half-life (Morris and Mathews 1989; Scott et al., 1991). PCNA has also been shown to be involved in DNA repair, which is not directly associated with proliferation (Celis and Madsen, 1986; Toschi and Bravo, 1988; Shivji et al., 1992; Wood and Shivji, 1997), which could also have contributed to the fact that a high proportion of cells were PCNA labelled compared to BrdU.

6.4.5. Apoptosis of the ovary

In the ovary the critical decisions between cell survival and cell death involve extensive communication between groups of pro-apoptotic and pro-survival

molecules. It is believed that this highly regulated coordination is entirely a quality control mechanism, supporting the viable oocyte with potential to ovulate and to eliminate those oocytes and follicles that become atretic. It is well documented that programmed cell death plays an important role in the regulation of ovarian maintenance with controlled cell death present at numerous stages of oogenesis and folliculogenesis. DAZL is undoubtedly essential for oocyte survival and by mechanisms not yet identified it may be contributing to the fate of the oocytes since, in the absence of DAZL, oocytes are lost after birth in mice. DAZL therefore could possibly be linked to factors initiating cell survival, including possible interactions with PDCD4. Thus we investigated the potential involvement of PDCD4 in ovarian apoptotic mechanisms, in both follicle atresia and luteolysis of the CL.

Alternative treatment regimes were designed to initiate follicle atresia by FSH withdrawal and to initiate functional luteolysis by the administration of two known luteolytic agents, bromocriptine and cloprostenol. FSH withdrawal undoubtedly induced follicle atresia in ovaries of d21 mice with extensive up-regulation of cleaved caspase 3 protein expression. As a result of this onset of apoptosis, cleaved caspase 3 mRNA was reduced following FSH withdrawal suggesting functional transcription had ceased and cell death was occurring. Even though PDCD4 has been targeted as a pro-apoptotic factor, induced upon apoptotic initiation, there was no difference in mRNA or protein expression in response to FSH withdrawal. One suggestion is that although PDCD4 was present in the cytoplasm in the developing follicle it remains functionally quiescent at the onset of rapid withdrawal of FSH as alternative death mediated pathways are recruited. Comparatively, follicle atresia is a quick and efficient process occurring rapidly within the ovary continuously whereas luteolysis is the gradual regression of the CL, which is both a functional and morphological process.

From the visual analysis of pro-estrous ovaries and after luteolytic treatment, it can be concluded that CLs are present at different stages of progression and regression with luteolysis occurring at different rates. PDCD4 phosphorylation (Palamarchuk et al., 2005) and hence translocation of functional protein to the nucleus may be

involved in the slow programmed cell death observed in the CL via shuttling between cytoplasm and nucleus. PDCD4 protein expression was remarkably different between CLs in the same ovary possibly due to the stage of regression of each CL. Not only did the intensity of cytoplasmic expression alter, nuclear expression was observed. Taking in to account that both old CLs from previous cycles and new CLs from the current cycle are present in the pro-estrous ovaries and not supporting pregnancy with their progesterone secretion, nuclear localisation of PDCD4 was inversely correlated with the expression of cleaved caspase 3. It became apparent that when PDCD4 was nuclear in the examined CL, cleaved caspase 3 expression was barely detectable. However when there was an increase in cytoplasmic expression and reduced nuclear expression of PDCD4, suggesting an increase in translation inhibition, there was a marked expression of cleaved caspase 3 cells within these regressing CLs. These observations suggest that caspase 3 is functionally required for apoptosis to proceed normally during luteal regression but that enhancement of PDCD4 within the cytoplasm may aid the onset of cleaved caspase 3 expression with both contributing to luteolytic degradation (Carambula et al., 2002). Extending this investigation into the inducement of functional and structural regression of the CL, by inducement of luteolysis, a similar pattern of PDCD4 and cleaved caspase 3 was observed. Again it was notable that there was increased intensity of PDCD4 both nuclear and cytoplasmic staining upon inducement of luteolysis whether by bromocriptine or cloprostenol emphasising a potential functional role in programmed cell death within the CL. As previously mentioned cleaved caspase 3 was only observed in CL in which there was a decrease in nuclear PDCD4 expression, and only in the CL from the bromocriptine and pro-oestrous models.

Finally, despite the original identification of PDCD4 mRNA as a potential target for DAZL mediated functions it has become evident that this novel transcription suppressor may be playing a significant role in luteolysis. The abundant expression and localisation of PDCD4 in the CL may be key finding in regulating and facilitating events within the CL of the mature ovary. One can now propose that there is a possible role for PDCD4 in programmed cell death associated with

structural CL regression. The observed difference in PDCD4 localisation from high cytoplasmic expression and nuclear localisation may be associated with the caspase cascade induction. In contrast, in follicles it has been shown that PDCD4 is probably not involved in cell death as no difference in protein or mRNA levels were detected as a result of natural or induced follicle atresia. The mechanisms and control of PDCD4 now require further investigation to explore the possible mechanisms of this protein and to determine whether PDCD4 plays a substantial role in preventing and controlling cell proliferation by means of mediated control over programmed cell death.

Chapter 4: DAZL target identification

4.1. Introduction

Careful characterisation of DAZ homologous sequences in a variety of animal species has led to the identification and classification of the "DAZ" gene family. This group of genes consists of three members: BOULE, DAZ-like (DAZL) and DAZ (Cooke et al 1996; Eberhar et al., 1996; Carani et al., 1997; Houston et al., 1998; Maegawa et al., 1999; Karashima et al., 2000; Xu et al., 2001). Members of this family are considered critical for germ cell development and are believed to be master regulators in germline gene expression, although they exhibit variations in phenotypic outcome depending on species and sex (Zeng et al., 2007). The human DAZ gene was originally isolated from the AZFc region on the long arm of the human Y chromosome which is often deleted in individuals who present with non-obstructive azospermia or severe oligospermia (Reijo et al., 1995). The biological function of this gene and its involvement in infertility remains unclear partly due to the absence of Y-link DAZ orthologues in mammals lower than the old world monkeys. Unlike the human, the murine homologue is autosomal and situated on chromosome 17, with expression exclusive to the male and female gonad (Cooke et al., 1996).

Attention has been given to the molecular interaction of DAZ proteins because of the presence of a highly conserved RNP-type RNA recognition motif, characteristic of DAZ proteins and indicative to RNA-protein interactions. A role for the DAZ-related proteins in translation regulation was first proposed by the identification of a genetic interaction in Drosophila between Boule and meiosis-specific CDC25 homologue, Twine (Mains and Wasserman, 1999). Furthermore, it has also been reported that DAZL can bind to the 5'UTR of CDC25c *in vitro* (Venables et al., 2001). In contradiction to these findings, CDC25a and not CDC25c and Tpx1 mRNA were shown to specifically interact with a GST-DAZL fusion protein (Jiao et al, 2002). In addition DAZL has also been shown to be associated with Pumilio2 (Pum2) a potential translation repressor (Moore et al., 2003). Subsequently, Sdad1 was selected as a candidate mRNA to which both DAZL and the DAZL-interacting

protein Pum2 bound simultaneously (Fox et al., 2005). Mouse DAZL additionally binds polyA+ RNA and has been shown to be associated with actively translating polyribosomes (Tsui et al., 2000), implying that DAZL may be functioning as an mRNA stabiliser and/or involved in translational regulation. In addition, members of the DAZ family have been shown to act directly to stimulate translation of RNAs bound through the recruitment of 80S ribosomes via an interaction with poly(A)-binding proteins (PABPs) (Collier et al., 2005). DAZL has also been suggested to play a role in the transport of specific mRNAs as it forms a specific interaction with the dynein light chain component of the dynein–dynactin motor complex (Lee et al., 2006). It has been recognised that TSSK 1, 2 and 4 3'UTRs bind to mouse and human DAZL (Zeng et al., 2008). TSSK genes are expressed almost exclusively to the mature testis and have been associated with regulatory event in spermatogenesis.

In addition to these protein: RNA interactions, DAZ family proteins can interact with a number of other proteins, many of which are themselves RNA binding proteins and are capable of forming hetero- and homodimers with other DAZ family members. These associated proteins include DAZAP1, DAZAP2, hQK3, DZIP1, DZIP2, DZIP3, (Ruggiu and Cooke, 2000; Tsui et al., 2000a; Xu et al., 2001; Moore et al., 2003, 2004; Urano et al., 2005; Lee et al., 2006). The functional significance of many of these protein interactions and mRNA targets remains to be clarified *in vivo*, although it seems highly probable that interactions with different proteins and mRNAs will have significantly different biological outcomes.

It is only recently that two studies have determined true *in vivo* DAZL mRNA targets, MVH and Sycp3, which were identified as co-immunoprecipitated to be directly associated with the endogenous DAZL protein (Reynolds et al. 2005; 2007). Firstly, MVH is a well recognised essential germ cell specific gene and it has been shown that germ cells of male DAZL KO mice (<d19 post partum) contain reduced levels of MVH protein, indicating that DAZL-mediated regulation of MVH translation is crucial for mammalian spermatogenesis. However, female DAZL KO mice are completely lacking in ovarian germ cells postnatally. Therefore germ cell specific MVH will be undetectable and it may be that completely different

mechanisms are in place between the two genders. Sycp3 is also recognised to be essential for meiosis (Yuan et al., 2002), specifically for the formation of the synaptonemal complex lateral element. In addition the Sycp3 knockout model displays a block in meiotic prophase which resembles the DAZL KO phenotype and in the DAZL KO male, Sycp3 protein expression is also reduced indicating that it may be regulated by DAZL. Furthermore, the absence of Sycp3 in the female mouse promotes aneuploidy in oocytes by inducing defective meiotic chromosome segregation (Yuan et al., 2002), again showing similar characteristic phenotype to the DAZL KO.

With reference to the KO DAZL model, whereby DAZL protein is absent, genuine *in vivo* DAZL targets would not be subjected to normal translational regulation. As demonstrated in Chapter 3, the Het animals also appear to undergo alterations in translational regulation, accounted for by the reduced gene expression which theoretically reduces protein production and hence, subsequent RNA binding function. In an attempt to identify targets one proposed suggestion was that a decrease in translation regulation due to DAZL function, could lead indirectly to a decrease in stability levels of target mRNAs (Jacobson and Peltz, 1996). This would result in differences in transcript levels between KO, Het and Wt animals. Hence the targets would have different mRNA expression levels, bearing in mind that not only is DAZL absent in the KO model, but so are the postnatal female germ cells.

Attempts to identify a consensus binding sequence for murine DAZL have produced conflicting results (Venables et al., 2001; Jiao et al., 2002), almost certainly reflecting the differences in experimental approaches taken and the multitude and complexity of *in vivo* interactions. The results from a study using a combined three-hybrid and SELEX approach, suggests the general consensus binding sequence as (G/CUn)n (Venables et al., 2001). Furthermore the results from a subsequent study which used recombinant GST-tagged DAZL in mouse testis extract, defined a 26 bp motif which was present in eight of the DAZL bound mRNAs (Jiao et al., 2002).

Despite the plethora of published DAZL mRNA targets and protein interactions, the function of DAZL still remains elusive. As mentioned, it is unclear whether comparable mechanisms are functioning between the male and female germ cells, and with the difference in meiosis occurrence in germ cell development the likelihood of altered regulatory control mechanisms is highly probable. Although target mRNAs have been identified, these results are biased to male spermatogenesis with limited data present from the female, now expanded by the studies in Chapter 3. In addition there are probably many targets yet to be determined. Therefore, the main aim of the current study was to explore the biological functions of DAZL during oocyte development exploiting techniques used to identify true *in vivo* targets. In this way it was hoped to identify how DAZL is functioning within the ovary *in vivo* and to identify the potential mechanisms which are contributing to the Het phenotype which in turn may aid the understanding of DAZ associated infertility problems.

4.2. Materials and methods

4.2.1. UV crosslinking immunoprecitpitation

The Ultraviolet (UV) cross-linking and immunoprecipitation (CLIP) method exactly as described previously (Ule et al., 2003; Renolds et al., 2005; 2007) was used to identify mRNAs associated with DAZL protein *in vivo* (Figure 4.1). Endogenous DAZL protein was immunoprecipitated from Wt and Het mouse oocytes, ovaries or testis homogenates and the co-purified RNA isolated. Control immunoprecipitations were carried out using an anti-DAZL antibody in the presence of the peptide to which it was raised (negative control) or using testis tissue homogenate looking at the MVH gene expression (positive control) (Reynolds et al., 2005). All work carried out on the DAZL mouse line required their genotypes to be known before any progression of experiments. Genotyping for wild type, heterozygous and knock out animals was achieved by PCR (Section 2.5).

4.2.2. Tissue preparation

Large pools of Wt and Het mouse oocytes (500) were manually dissected from d21 ovaries in Hanks Balanced Salt Solution (GIBCO) on ice before cross-linking three times with 4000 μJ of UV (CL-1000 Ultraviolet Crosslinker (UVP, Amlab)). The suspension was agitated thoroughly between each irradiation. In addition, intact Wt and Het d10 ovaries were also used as they contain a greater number of small primary and primordial follicles expressing higher or more concentrated levels of DAZL protein (eight ovaries were grouped for each sample). Testis from adult DAZL mice were used as a positive control, the testis was removed from its bursa and the seminiferous tubules teased apart to form a thin layer, thus exposing a large surface area prior to crosslinking.

The direct irradiation from UV light forms covalent bonds between proteins and mRNA which are in direct contact (DAZL protein: mRNA target). It is important to mention that the efficiency of this reaction is low but compensated for by the subsequent PCR reaction amplification in the final stages of this pulldown. The covalent bond formation between the protein and mRNAs of interest allows the application of rigorous purification procedures therefore obtaining highly purified

complexes between the protein and the target mRNAs. In addition cross-linking allows partial digestion of the RNA while retaining the core element involved in protein binding.

4.2.3. Immunoprecipitation

Following cross-linking the samples were centrifuged at 6000rpm for 2 minutes. Aliquots of supernatants were taken for Western blot analysis at each stage of the experiment to confirm pull-down of the correct protein, DAZL. The formed pellet was resuspended in 1x PXL (1x PBS containing 0.1% SDS, 0.5% deoxycholate, 0.5% NP-40) before being homogenised by a hand held rotary homogeniser (Sigma). The samples were then spun in a micro-centrifuge at 13000rpm for 10 minutes at 4ºC and a further sample of supernatant taken for Western analysis along with the pellet. Only the supernatant was used for the subsequent preclear steps. For each 1 ml of supernatant (adapted accordingly) 1µl of RNaseguard (Ambion), 10µl of DNAse 1 and 100µl Protein A sepharose (PAS) (vol:vol PXL and PAS-rinsed 2x) was added. The cocktail was then incubated for 1 hour at 4ºC on a rotary wheel to eliminate any endogenous IgGs which may interfere with the antibody. Samples were then centrifuged at 4000rpm for 2 minutes at 4ºC. The sample was subsequently split into two aliquots of approximately 500µl and immunoprecipitation of proteins was then carried out. One aliquot was incubated with the purified monoclonal antibody to murine DAZL (D) or with the antibody in the presence of the peptide (P+D), to which it was raised, along with PAS beads (Table 4.1). The samples were then incubated at 4ºC overnight on a rotary wheel.

Table 4.1: Immunoprecipitation of tissue and DAZL antibody

	D	**P+D**
Anti DAZL antibody	20µl	20µl
PAS 50%	50µl	50µl
6µg/ul peptide	-	10µl

Following overnight incubation the samples were centrifuged at 4000rpm at 4°C for 1 minute and the supernatant removed. The PAS beads were washed 3x in 500µl ice cold 1xPXL, and 1x in 500µl of PK (PXL + 500mM NaCl) with each sample being centrifuge at 4000rpm at 4°C for 1 minute between each of the washes. The beads were then treated with 100µl final volume of PK + 2.5µl Proteinase K (enzyme) (Ambion), and incubated for 20 minutes at 37°C.

4.2.4. RNA isolation

After incubation of the beads with PK and Proteinase K, 100µl of RNA phenol was added to the mix in a fume hood before being vortexed thoroughly for 2 minutes, followed by centrifugation at 13000 rpm for 5 minutes at room temperature. 80µl of the aqueous phase (top layer) was then removed and stored on ice. 100µl of RNase free H_2O was then added to the solution, before being vortexed and spun at 13000 rpm for 5 minutes. 80µl aqueous phase of this second extraction was then removed and added to the previously collected 80µl. This 160µl RNA sample was kept on ice and 10µl Linear acrylamide (Ambion), 160µl EtOH and 160µl isopopanol were added and left to precipitate at -20°C (dry ice) for 10-30 minutes. The RNA samples were then centrifuged at 13000 rpm for 30 minutes, washed with 75% EtOH and centrifuged again at 13000 rpm for 20 minutes, before being dried and resuspended in RNase free dH_2O (20µl). The RNA then underwent DNase treatment (Ambion) by adding 2µl 10x buffer and 1µl of DNase and incubated at 37°C for 20 minutes. After treatment, 2µl of inactivating reagent (supplied) was added and the sample agitated for two minutes.

RNA was quantified using the Nanodrop (Section 2.3.3) before being reverse-transcribed using a sensiscript cDNA kit (Qiagen) (Section 2.3.4), followed by PCR to determine relative levels of transcripts present (Section 2.3.6). PCR primers were designed against the 3' end of the target gene and a mock reverse transcription reaction was carried out in all cases as a control for contamination with genomic DNA. Gene-specific primers for MVH were used as a positive control reaction (Table 4.2).

Table 4.2: Primer Sequence for 3' end of MVH

Target Name	3' sequence	5'sequence
MVH*	AggCCTgggggAAATgTgTTTCATCTTTT	TAACTAgTgAATAgTgTATCAgTACAATgTAAT

*gifted by N.Reynolds

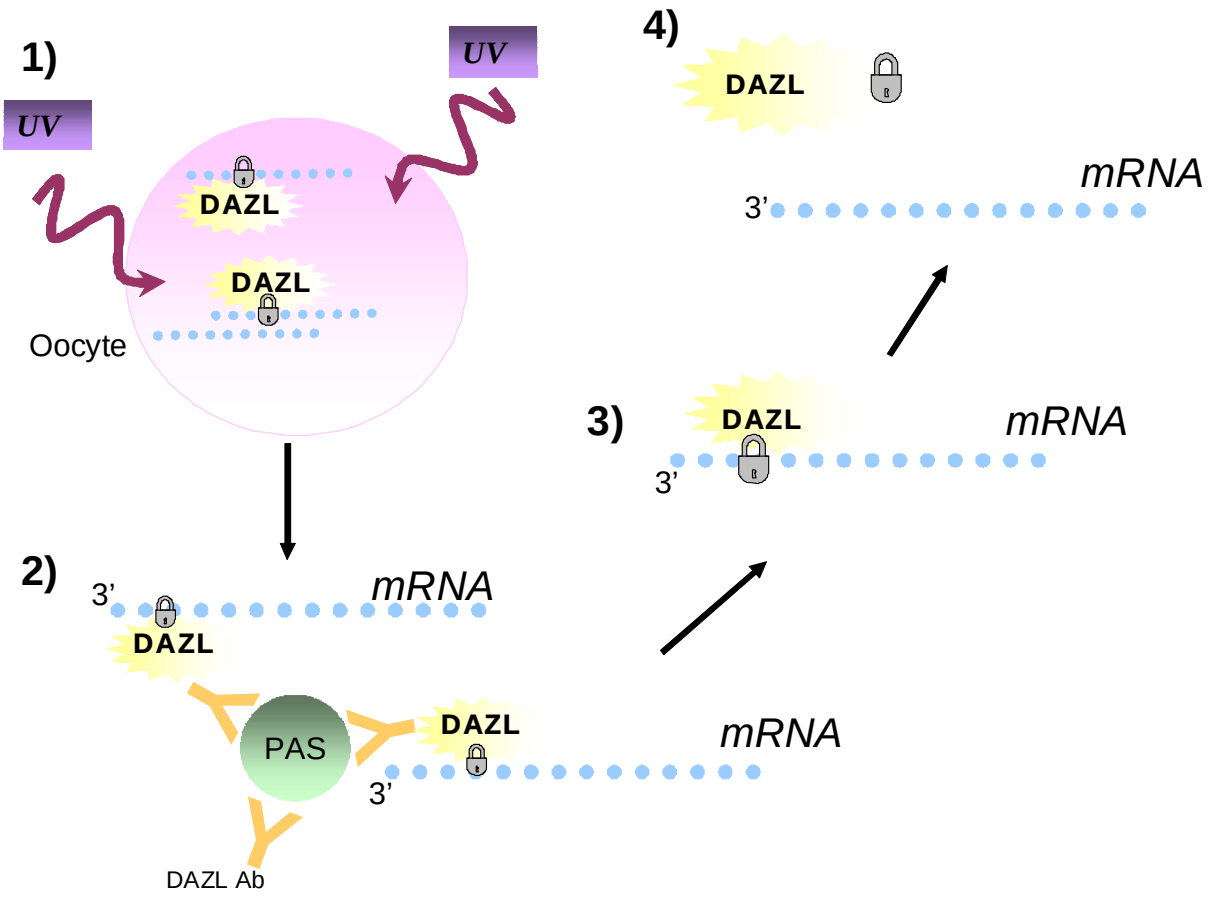

Figure 4.1: Schematic summary diagram of the UV CLIP method, demonstrating 1) UV cross-linking of protein (indicated by padlock): mRNA interactions within the oocyte 2) PAS beads used to pulldown mRNA linked to DAZL protein which in turn absorbed by the DAZL antibody which is attached to the PAS, 3) Dissociation of protein: RNA complex from the Antibody and PAS and 4) RNA extraction and identification of target mRNAs.

4.2.5. Protein detection

Western blot analysis was performed on sample extracts and tissues (Section 2.6). After incubation of immunoblots with primary and secondary antibodies (see individual results for concentrations) the blots were scanned using the Licor.

Immunohistochemistry was performed using the standard protocol (Section 2.7; 2.8).

4.3. Results

4.3.1. Immunoprecipitation of DAZL/mRNA complexes

To determine the putative targets to which DAZL protein binds within the ovary coimmunoprecipitation was performed. The technique (Reynolds et al., 2005; 2007) used had not been previously applied to ovarian tissue therefore initial investigations replicated the same immunopulldown protocol that detected MVH and SYCP3 within the testis (Reynolds et al., 2005; 2007). To increase the probability of extraction of mRNAs bound to DAZL protein, and because DAZL protein is concentrated within the oocyte cytoplasm, oocyte preparations were initially used. The *in vivo* target, MVH was also selected for initial attempts to identify DAZL interactions within the ovary since this was achieved from the testes (Reynolds et al., 2005) and oocytes express easily detectable levels of MVH (Chapter 3). The original proposal was to link the findings from the extensive bioinformatic *in silico* trawl (Chapter 5) using the putative DAZL binding motif (Venables et al., 2001) in conjunction with UV cross-linking immunoprecipitation to identify *in vivo* targets within the ovary. Optimising, repeating and applying this technique to oocytes could determine whether DAZL binds the same targets as in the testis or whether it functions through different mechanisms.

4.3.2. MVH detection in testis extracts

Control experiments were initially performed whereby endogenous DAZL protein was immunoprecipitated from mouse testis homogenate and any co-purified RNA isolated. Within experiment control immunoprecipitations were also carried out using the anti-DAZL antibody in the presence of the peptide to which it was raised (negative control). The results from the control pull down verify the results of Reynolds et al. (2005), whereby MVH transcripts were detectable after the immunopulldown (Figure 4.2).

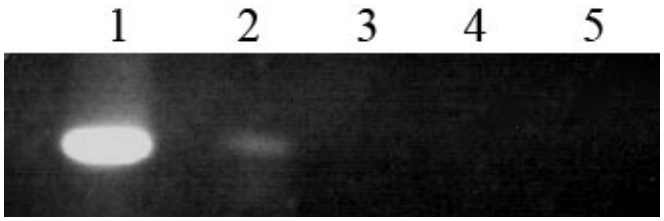

Figure 4.2: Photomicrograph of PCR products separated on agarose gel electrophoresis demonstrating UV CLIP protocol worked in the testis detecting MVH. Band in lane 1) testis positive control for MVH mRNA expression 2) CLIP result showing MVH mRNA was pulled down with DAZL antibody 3) MVH absent when blocking peptide was used with the antibody 4) RT –ve and 5) H_2O blank.

4.3.3. Immunopulldown with oocyte and ovary preparation

Despite numerous attempts (oocyte n=4; ovary n=4) this technique proved to be unsuccessful with the oocyte preparations even when using an abundant supply of DAZL expressing oocytes from eight x d10 ovary preparations. The initial sample preparation of oocytes presented spurious RNA results, with a peak detected at the wrong wavelength suggestive of either DNA or reagent contamination or possibly no RNA. In addition other potential problems include, the fact that the oocyte pools collected were from larger follicles and from the immunohistochemistry (Figure 4.3) oocytes from d21 follicles did not show abundant in DAZL protein expression. When analysing DAZL protein expression in d10 ovaries, it was obvious that a more homogeneous population of positively stained oocyte were present. It may be that DAZL protein is too low in the ovary and this immunoprecipitation method cannot be applied in the same way as that which worked for the testis. Furthermore it may be that the DAZL antibody is not working efficiently in the pulldown with ovarian tissue, in contrast to the testis, and that the sensitivity of the technique is not compatible when using ovarian tissue.

4.3.4. Specificity of DAZL antibody

To assess the specificity of DAZL protein expression the commercially available monoclonal antibody from Abcam (Cambridge, UK) clone 311/A (original

N.Groome Ab clone 311/A) was used. Immunohistochemistry was performed on the testis of an adult male mouse and ovaries from d10 and d21 female mice (Figure 4.3). Immunohistochemisty confirmed that DAZL is expressed at the protein level in germ cells of the testis (Figure 4.3(a)), the small primordial follicles of the d21 (Figure 4.3 (b)) ovary and more abundantly in the small primordial follicle of the day 10 ovary (Figure 4.3 (c)). DAZL protein expression was present but reduced in the oocytes of the larger pre-antral follicles.

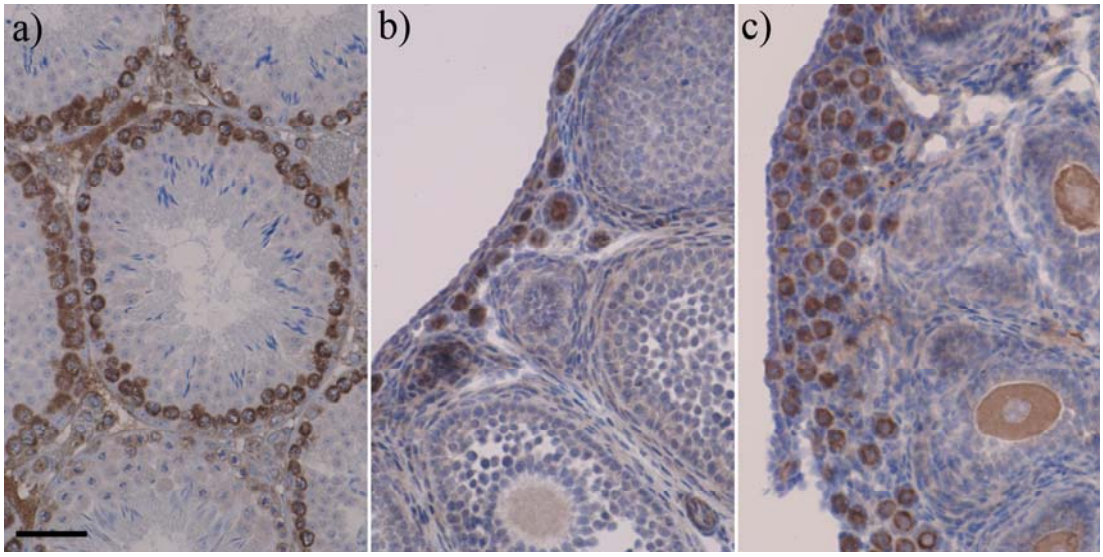

Figure 4.3: Photomicrograph of immunohistochemistry for DAZL protein staining in (a) adult mouse testis (b) mouse d21 ovary and (c) d10 mouse ovary. Scale bar represents 50μm.

4.3.5. Western Blot problems and material limitations

Following the unsuccessful immunopulldown using d21 oocytes and d10 ovaries a decision was made to look at protein expression using western blot analysis of the pull-down washes. This was done to verify that the antibody was functioning in detecting the correct protein, and checking at each stage of the procedure that the PAS and antibody were working in the correct binding complex. As no protein was detected the results are not presented.

After discussions with Nicola Reynolds (HGU, Edinburgh) a further decision was made that in order to obtain optimal results with the immunopulldown from ovarian

tissue, there had to be an abundance of DAZL protein in the initial tissue sample, to ensure a detectable outcome was achieved. Therefore, the DAZL protein from the ovaries must be detectable on a western. A selection of ovarian samples were used and as a result of the limited protein extracted from the samples, detection of the oocyte protein was limited on western blots (Figure 4.4). Furthermore, the protein bands detected on the Western blot (Figure 4.4) did not coincide with the published molecular weight of DAZL, where the size ranges from 33-35kDa while the band detected on the Western blot was approximately 50kDa (beta-tubulin was the correct size at 50kDa). One possible suggestion was that the DAZL antibody was detecting the DAZL protein as a heterodimer on the Western blot, with a list of potential associated proteins shown in Table 4.2.

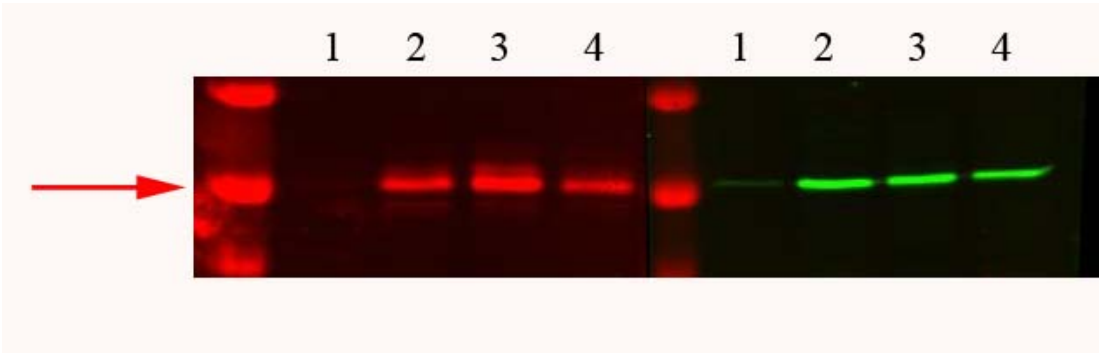

Figure 4.4: Western immunoblot of DAZL protein expression (red) and beta tubulin (green) as a loading control. Faint positive band present in Lane (1) oocyte (6µg) whereas (2) Het d21 ovary (10ug), (3) 2 x d10 Het ovary (10µg) and (4) 2 x d10Wt ovary (10µg) all have a strong positive DAZL protein bands consistent with beta tubulin protein expression. The red arrow indicates the size of the "DAZL" protein detected.

Table 4.3: Proteins which interact and form heteodimers with DAZL.

Protein	**Size (kDa)**	**Reference**
Boule	32	Xu et al., 2001
DAZAP1 DAZAP2	43 17	Tsui et al., 2000
Pumilio2	140	Fox et al., 2002

No other bands were detected on the Western blot. Thus to determine whether the band detected was indeed DAZL ovaries from KO mice were used since DAZL protein should not be produced due to the transgenic modification. Different DAZL genotypes, in conjunction with male and female tissue, were used with the hypothesis that the KO animals would provide a negative result. The results show (Figure 4.5) that all genotypes exhibit the protein band at 50kDa and with two protein bands detected only in the adult testis, the lower one being of the correct size, 33kDa, with no DAZL protein detectable at d6 in either Wt or Het testis.

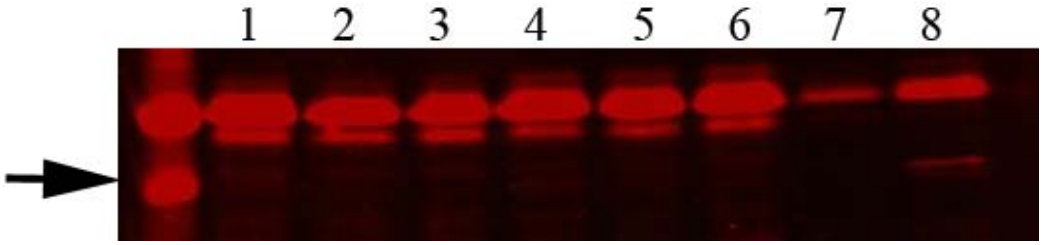

Figure 4.5: Westen Blot analysis of DAZL protein expression from tissue from (1-3) 2 x d6 female ovary (4-6) 2 x d6 testes male with 1 and 4 Wt, 2 and 3 Het and 3 and 6 KO and 7 adult female KO ovary and 8 adult testis Wt. Arrow indicates DAZL protein band in lane 8 in the mouse testis.

Further to the investigation, a screen of male and female tissues was performed and once again all tissues (Figure 4.6) produced a positive band at the 50kDa size with only testes having a detectable band at 33kDa in lane 14.

Figure 4.6: DAZL protein expression in male and female organs. Western blot image of (1-6) d6 male and (7-12) d6 female; lanes 1+7 adrenal, 2+8 spleen, 3+9 heart, 4+10 kidney, 5+11 lung, 6+12 liver, 13) adult Wt ovary and 14) adult Wt testis. Red arrow indicates positive band in the testis and the possible faint band present in the heart (3 and 9) of the male and female (20µg protein).

DAZL protein has been previously detected in human fetal ovary and testis by Western analysis (Anderson et al., 2007) and the 50kDa band was not observed. Therefore, a fetal human testis sample was used as a comparison to attempt to identify the DAZL protein in the mouse ovary (Figure 4.7).

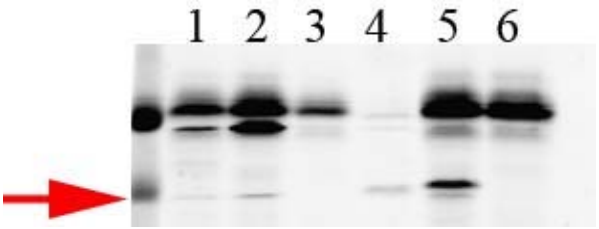

Figure 4.7: Western Blot analysis of DAZL protein expression in (1) male mouse liver; (2) heart; (3) ovary; (4) human fetal testis; (5) mouse testis Wt and (6) mouse testis KO (20µg protein).

Interestingly, a positive band of the correct size was detected in the heart and liver for DAZL when the protein concentration was increased, with a single band present in the human fetal testis of the correct size with only a slight 50kDa band when compared to the mouse tissue. A strong positive band is present in the Wt testis along with a band of the correct size, only the 50kDa bands are present in the KO.

To further elucidate the detectable bands at 50kDa all tissue samples were incubated with either secondary antibody only or IgG control to ablate any none specific

primary antibody interactions. The findings illustrate that in the absence of the DAZL antibody the positive protein bands of the correct size of 33 kDa are absent but the protein bands detected at the higher molecular weight 50kDa are still present (Figure 4.8). These results suggest that the bands present at 50kDa are non-specific products of the secondary antibody.

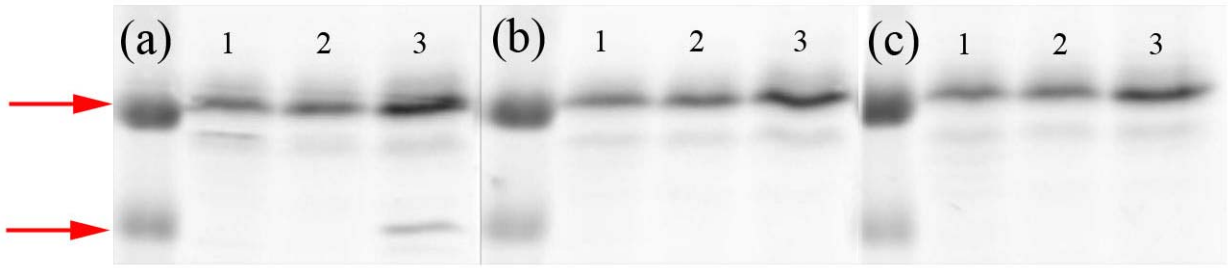

Figure 4.8: Western Blot analysis of DAZL antibody verification. DAZL protein expression in the presence of (a) primary antibody, (b) no primary but secondary (c) no primary but IgG mouse instead and secondary. 1) kidney 2) liver 3) testis at 20µg protein. Red arrows indicates protein bands at 50 kDa and 33kDa

To further elucidate the problems associated with the secondary antibody, different secondary antibodies were used to eliminate the chance that this result is only specific to the Odyssey goat anti-mouse secondary (Figure 4.9). The other secondary antibodies used are listed below:

- Rockland IRDye 800 Conjugated Affinity purified anti mouse IgG (H+L) (goat) (Figure 4.9 a, d)
- Molecular Probes Alexa Flur 680 goat anti mouse IgG (Figure 4.9 c, e)
- Odyssey goat anti mouse 680 (1:5,000) (Figure 4.9 c, f)

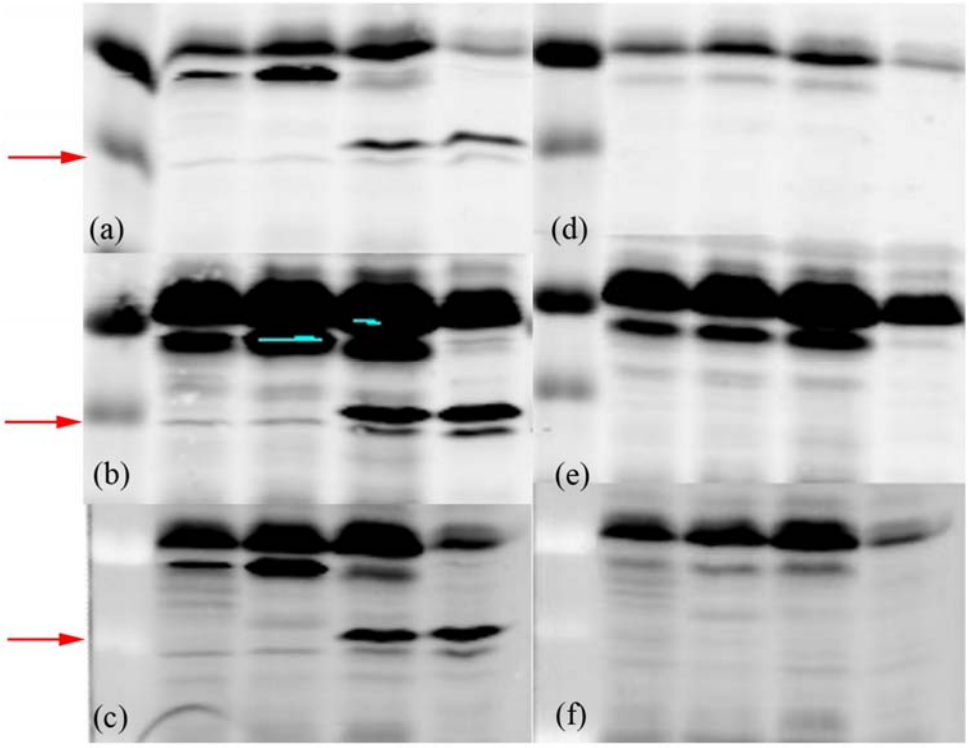

Figure 4.9: Western blot analysis of different Secondary antibodies (on previous page). Panels (a), (b) and (c) incubated with primary DAZL and secondary antibodies. (d), (e) and (f) incubation with secondary antibody only. Protein from lane 1) liver 2) heart 3) d10 testis 4) adult testis (20µg protein). Red arrow represents DAZL protein 33kDa.

The results confirm that the observed bands at 50kDa band are a secondary antibody binding artefact, as they are still present in the absence of the primary antibody (Figures 4.8 and 4.9). The presence of the DAZL antibody is required to detect DAZL protein bands of correct size. After the detection of not only DAZL protein in the mouse and human testis, antibody specific proteins were detected in the liver and heart samples. A further tissue screen was performed using increasing protein concentrations of 20µg, 40µg, 60µg (Figure 4.10). Two protein bands were present in the testis sample, one at approximately 30kDa and the more prominent band at 33kDa. This secondary band which was present in the testis sample corresponded to the protein band detected in the different tissue samples, the liver, kidney and heart but not the brain. Increasing the protein concentration of the input sample increased the intensity of the detectable protein.

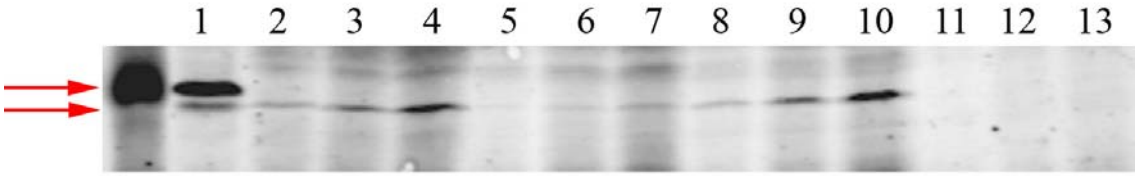

Figure 4.10: Western Blot analysis of mouse tissue for DAZL protein (33 KDa). 1) positive control testis, 2-4) liver 20ng/40ng/60ng; 5-7) kidney 20ng/40ng/60ng; 8-10) heart 20ng/40ng/60ng and 11-13) brain 20ng/40ng/60ng protein. Top red arrow indicates DAZL band 33kDa, lower red arrow indicated protein band detected in all tissues apart from brain, approximately 30kDa.

4.3.6. Immunohistochemistry analysis of DAZL protein expression

To further investigate the findings of the positive DAZL protein band present in the liver, kidney and heart in the Western analysis, immunohistochemistry using Wt and KO mouse tissue was performed (Figure 4.11). Interestingly very specific heapatocyte cells which surround the blood vessels within the liver showed positive DAZL protein in both the Wt and KO tissue samples. There was brown staining detected within the heart and kidney samples but due to the nature of these tissues and despite the negative controls being clear these results may be non-specific. All negative controls and IgG controls (not shown) were negative. These results suggest that the protein band detected in the western analysis and immunohistochemistry is not DAZL as it is present in the KO tissue samples. However it does suggest that the monoclonal antibody used is possibly detecting a related protein and this requires further analysis.

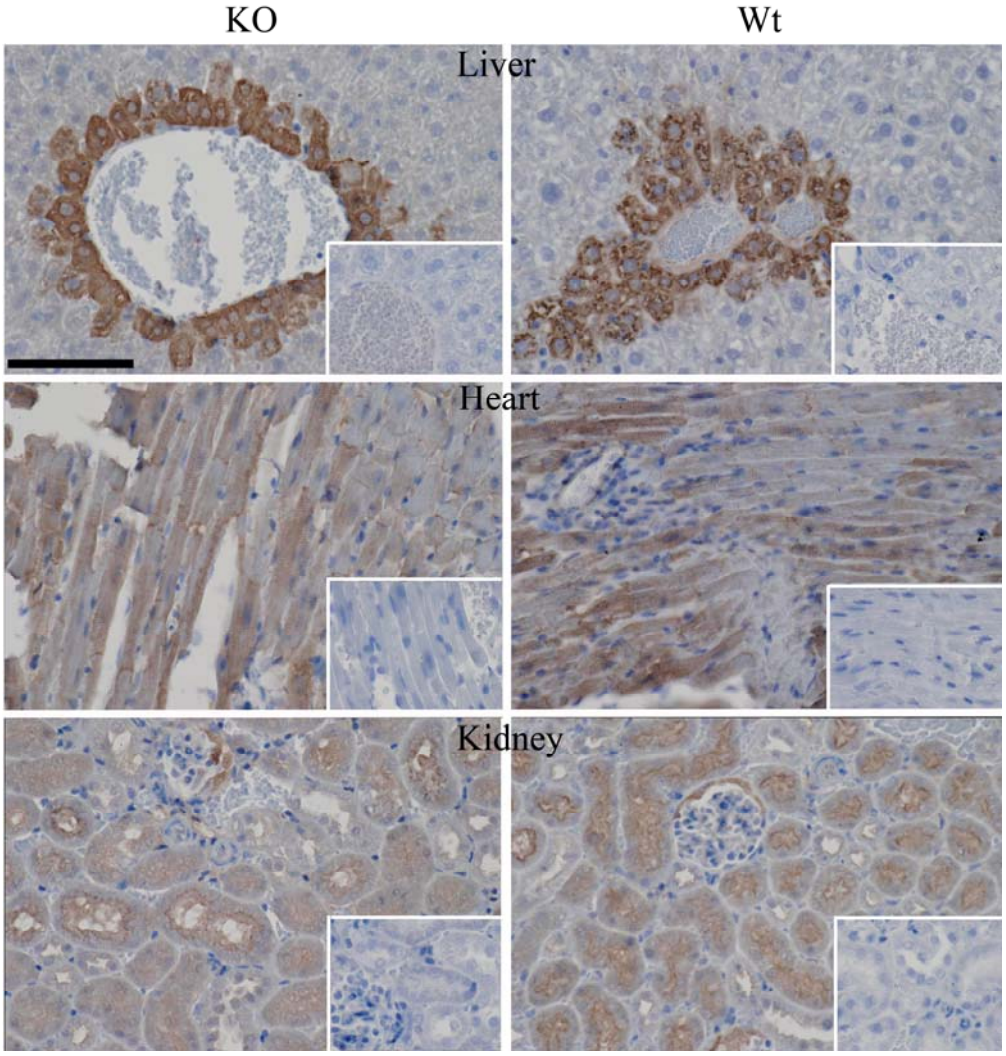

Figure 4.11: Immunohistochemistry of KO and Wt liver, heart and kidney. Brown positive specific staining located around the blood vessels of the liver, in the muscle fibres of the heart and within the tubules of the kidney. Insets are negative antibody controls (Scale bar represents 50µm).

4.3.7. DAZL mRNA expression tissue screen

The next step was to determine if mRNA was present within the tissue of interest which had shown DAZL protein expression by both Western and immunohistochemistry. Results from the RT-PCR demonstrated that the mRNA transcripts for DAZL were greatest in the testis and ovary (Figure 4.12, lane 1 and 2). There was evidence of mRNA DAZL transcripts present in the screen of different mouse tissue and although not detectable at the same intensity as the ovary and testis

this screen provides subsequent evidence that there may be a similar protein to DAZL present in different tissues apart from the reproductive organs.

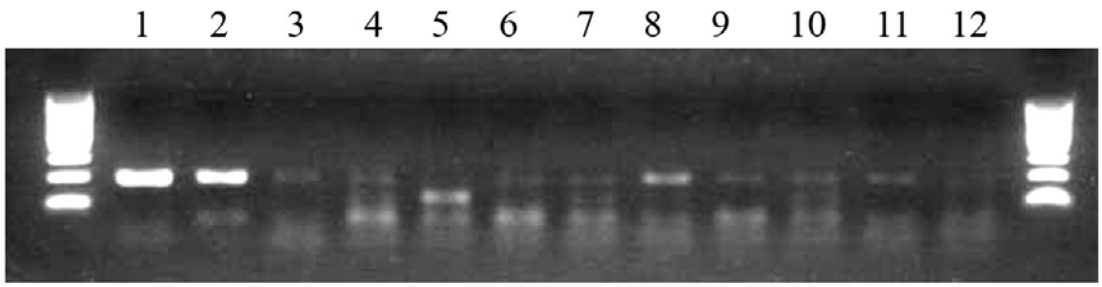

Figure 4.12: PCR photomicrograph of tissues expressing DAZL mRNA transcripts from d10 animals 1) testis 2) ovary 3) heart 4) liver 5) spleen 6) kidney 7) lung 8) adrenal and from d21 animals 9) liver 10) spleen 11) kidney and 12) lung.

These findings of "DAZL" expression at the mRNA level by PCR analysis in addition to the protein detection by Western blot and immunohistochemistry require further extensive investigation with careful interpretation.

4.4. Discussion

The major aim of these investigations was to develop and optimise the UV CLIP methodologies used to identify testis DAZL targets *in vivo*, and apply them to the ovary. After extensive trials using DAZL expressing oocytes and extending this to d10 ovaries which have a higher intensity of DAZL protein present in the oocytes of the primordial and primary follicles, and using MVH as a potential target mRNA it became apparent that the application of this technique to the ovary was problematic. Abundant DAZL protein is available from the male germ cells within the testis, whereas DAZL protein was undetectable by Western blot from oocyte and ovary samples. One of the possible limiting factors in the CLIP analysis of the ovary was the availability of DAZL protein as replicate results were obtained by performing a testis positive control pulldown to identify MVH. Furthermore, the previously identified targets were originally detected from a comparative microarray which identified fold difference in transcript expression between Wt and KO testis (Reynolds et al., 2005; 2007). Of a number of potential targets MVH was selected for further investigation and latterly SYCP3, both of which have now been confirmed to bind *in vivo* with testis DAZL. In particular the putative MVH target detected within the testis preparation may not be a target of DAZL within the ovary as there are difference in meiotic activity between male and female germ cells. Meiosis of the germ cells begins in the fetal ovary but in the adult testes and it is possible that targets will vary between genders. Therefore using MVH may only be of valuable as ensuring that the validity of the CLIP technique in the testis and MVH may not be a DAZL target interacting in oocyte.

The investigation using Western blots into DAZL protein expression within the mouse ovary after failure of the CLIP, suggested that, DAZL protein was undetectable by Western analysis in the samples used, despite being detected by immunohistochemistry. The results from this also directed further investigation into a higher molecular weight protein band detected by the DAZL. After systematic investigations using different tissues, protein concentrations and primary and secondary antibodies, this higher molecular weight protein was shown to be an artefact of the secondary antibody binding to the membrane.

Furthermore, the investigation into DAZL protein expression identified a subsequent protein, possibly DAZL, or similar homologue or isoform in other tissues as well as the testis. This protein was of similar size at approximately 30kDa as DAZL 33kDa but detected in the heart, liver and kidney of d6 and d21 Wt male and female samples. To verify this, the peptide sequence to which the monoclonal antibody was raised was used to perform a BLAST sequence match search and the outcome of this confirmed that the antibody epitope does not match the portions of the DAZL gene which are removed as a result of the mutation process. This supports the results from the immunostaining where there is no evidence of DAZL protein in the KO ovary, but this is not surprising as there are no oocytes present. These results are contradictory and although DAZL should definitely not be present in the KO or other somatic tissue, protein and mRNA specific for DAZL or similar a protein sequence were detected by Western blot and PCR from KO tissue.

Supporting the localised expression of DAZL to the germ cells within the gonads, it has been previously shown (Cooke et al., 1996) by RT-PCR that the DAZL gene expression is solely confined to the gonads of adult mouse and transcripts are not detected in any other tissues. Studies using Xenopus have additionally demonstrated that by Western analysis, protein is only detected in the testis and ovary (Mita and Yamashita, 2000). In the Medaka fish oDAZL was not detected in somatic tissue (Xu et al., 2007) by Northern blot analysis. A further report showed that murine DAZL was not been detected in the brain, lung, spleen or kidney (Maiwald et al., 1996). However, in the human DAZL transcripts have been detected in the amniotic fluid (Stefanidis et al., 2007), and in the CL, with the concentration decreasing with advancing luteal phase (Pan et al., 2002).

From the evidence above and from the results from the current studies the detection of “DAZL” protein in the KO or other tissue is not supported. One can suggest that the protein detected in these tissues is not DAZL but a protein with a similar sequence to which can be recognised by the DAZL antibody, possibly a different isoform. The identification of any such protein requires thorough investigation.

The KO phenotype has very different consequences for the male and females as does the heterozygous phenotypes due to the comparative variations present in germ cell development. In the KO, germ cells can complete mitosis, and embark on functional differentiation but thereafter, in both sexes progression through meiotic prophase requires the DAZL protein (Saunders et al., 2003). As previously mentioned the KO males have failure of spermatogenesis as germ cells are unable to complete the first meiotic prophase in the initial wave of spermatogenesis and subsequently germ cells decrease in number due to a block at the A-aligned to A1 transition, although it has been demonstrated that mitotically active germ cells can be present up to and including day 19 post partum. The Het males do have germ cells present and are fertile, yet spermatogenesis is severely compromised. There is evidence that sperm are highly defective and that the number of germ cells are reduced (Ruggiu et al., 1997). In the female the KO ovary does not contain any germ cells after birth, with the heterozygous presenting with enhanced fertility producing larger litters. This phenomenon is discussed later in Chapter 7.

So not only the lack of DAZL gene expression leads to the phenotypic differences but the loss of or reduction in its translation of target mRNAs may contribute to the phenotypes observed in DAZL KO and Het mice (Reynolds et al., 2005). It is also highly possible that as specific translational requirements change throughout germ cell development, the components and functions of the DAZL associated complexes may also alter (Reynolds et al., 2007).

This study was unsuccessful in establishing oocyte *in vivo* targets due to the limitations presented with using oocytes and ovarian tissue as the source of DAZL expressing germ cells. Therefore, the natural substrates of DAZL remain undefined within the ovary using the current CLIP technique (Reynolds et al., 2005; 2007) which was successful in detecting *in vivo* DAZL mRNA targets within the testis. The one major problem is the difference in germ cell distribution between the male and female, with oocytes being deficient in providing adequate DAZL protein to subsequently detect bound mRNAs. As previously mentioned the entire mechanism of control may well be different between male and female germ cells. Therefore a

reassessment of the approach to identify DAZL targets *in vivo* within the oocyte is required. One suggestion is to use a collection of fetal ovaries to increase to concentration of DAZL protein for the immunopull down although these oocytes will still be in germ cell nests and not assembled as primordial follicle, so again function may be different. In addition performing an age specific comparative microarray analysis on oocyte gene expression in an attempt to identify transcripts may show alterations in their transcription in comparison with the KO genotype and may provide some answers. Another problem arises when trying to identify how the target translation affects fertility of the Het females, considering how the oocyte may be pre-programmed or how functional activity may be altered, this has been investigated further in Chapter 3 with regards to the oocyte and Chapter 7 with regards to the follicle.

This study has been extremely helpful in illuminating problems with CLIP which arose as a result of using tissue with low abundance and expression of the target protein. Despite the failure to identify murine female DAZL mRNA targets it has transpired that DAZL or similar protein(s) may be present specifically within the liver. It is more likely that the antibody is detecting a related or similar protein and this requires extensive follow up investigations. In conclusion, to address the problems in the female germline associated with DAZL, highly complex studies are now required to target different developmental periods and stages of oocyte maturation and follicle formation to identify true *in vivo* targets of the DAZL proteins in the ovary.

Chapter 5: *In silico* identification of DAZL targets

5.1. Introduction

The presence of the RNA binding domain within the DAZL protein suggested its involvement in RNA metabolism, however the mRNAs to which the DAZL protein binds and functions within the oocyte remain to be elucidated. Diverse approaches have been adapted previously and utilised to identify the mRNA species that are specifically bound to this germ cell specific protein. In Chapter 3 a direct approach was used to elucidate potential oocyte affected genes by the heterozygous genotype and the results suggested that the overall function of the oocyte within the somatic cell complex is altered. In addition in Chapter 4 studies attempted to apply the successful techniques used to determine *in vivo* DAZL targets in the male and utilise them to identify targets within the oocyte. However this method gave inconclusive results and requires further optimisation but it did identify potential problems with the specificity of the DAZL protein and mRNA. What is known so far regarding potential DAZL targets fails to clearly define the importance, function and mechanisms involved with DAZL protein expression in the oocyte, and as previously mentioned all but two studies fail to show any *in vivo* interactions (Reynolds et al., 2005; 2007). In addition there are two unrelated published binding consensus sequences (Venables et al., 2001; Jiao et al., 2002) which have individually identified a range of target mRNAs that are bound at both the 5' and the 3' ends, suggesting that there may be multiple functions and mechanisms of action of this protein.

This chapter focuses on using a further approach, not previously utilised, to undertake the arduous task of identifying oocyte specific targets. The consensus sequences T2(G/C)T3-10(G/C)T2, which represents U rich regions of mRNA interspersed with G and C (Venables et al., 2001) was used in conjunction with an extensive bioinformatics *in silico* trawl of the mouse genome to identify DAZL mRNA transcripts. The outcome of this approach was a shortlist of oocyte specific putative DAZL target mRNAs.

5.2. Materials and methods

As a strategy to identify putative DAZL targets the National Centre for Biotechnology Information (NCBI) public database was used in conjunction with the 32 permutations of the Venables et al., (2001) consensus sequence for DAZL.

5.2.1. Sequence alignment

Established in 1988 as a national resource for molecular biology information, NCBI creates public databases, conducts research in computational biology, develops software tools for analyzing genome data, and disseminates biomedical information. These resources aid the better understanding of molecular processes which affect human health and disease. The consensus sequences were used to perform an *in silico* Basic Local Alignment Search Tool (BLAST) search (http://www.ncbi.nlm.nih.gov/BLAST/) (Figure 5.1). BLAST finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches.

Figure 5.1: Screen shot of the BLAST webpage

The identification of targets using this approach will not only be useful in potentially providing novel targets but also exploits the potential of the bioinformatics tools in recognising gene targets. The initial trawl required systematically selecting and identifying the permutations of the DAZL consensus sequence within specific mouse mRNAs. Using the BLAST software and selecting the database called "refseq_rna" which represents the mRNA sequences from NCBI Reference Sequence Project, and limiting the entrez query to *Mus musculus* short sequences with near or exact matches were identified. From these criteria a number of hits were produced, with information for the first 100 sequences and nucleotide alignments only for the first 50 sequences. It was therefore decided to set the limitation criterion to the first 50 sequences which were aligned and select these for further investigation. Once gene sequences were detected and checked to determine that the orientation of the putative binding domain was 5' to 3', the target sequences were matched and expanded if appropriate bases lay in an adjoining sequence. A data spread sheet was developed using Microsoft Excel to record the results from the initial *in silico* trawl.

5.2.2. Expressed sequence tags (ESTs)

An EST is a short transcribed nucleotide sub sequence intended to identify transcripts of genes. The use and identification of ESTs are instrumental to biological research and with approximately 37 million ESTs now available in public databases (e.g. GenBank 7/2006). The production of an EST occurs by a one-shot sequencing of a cloned mRNA (sequencing several hundred base pairs from an end of a cDNA clone taken from a cDNA library). The outcome of the cloning results in relatively low quality fragments whose length is limited by current technology to approximately 500 to 800 nucleotides. The sequence of nucleotides is complementary to mRNA and hence represents a proportion of the expressed gene (http://www.cambridge.org/catalogue/catalogue.asp?isbn=9780511166563&ss=exc).

Hits from the BLAST search were firstly individually compared against the mouse EST libraries using Unigene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD =search DB=unigene) (Table 5.1). If ESTs had been identified to either the oocytes

or the unfertilised ovum tissue groupings, they were selected and analysed in a further refined database.

Table 5.1: Mouse EST libraries

Lib ID	Library Name	Sequences
Oocyte		
Lib.18552	Eppig Hampl Solter fully-grown mouse oocyte	18790
Lib.16177	NIH_MGC_256	4487
Lib.16178	NIH_MGC_257	4263
Unfertilised Ovum		
Lib.10029	NIA Mouse Unfertilized Egg cDNA Library (Long)	13201
Lib.14142	NIA Mouse Unfertilized Egg cDNA Library (Long 1)	6443
Lib.1389	Mouse unfertilized egg cDNA	3098

Not Shown: 1 library having fewer than 1000 sequences

Once all genes had been subjected to an EST library search, the next stage was to use publications of oocyte expressed genes to confirm that ESTs were within the oocyte active genome. The two papers selected were chosen due to the extensive published lists of oocyte genes:

Stanton J. L., Green D. P. (2001) A set of 840 mouse oocyte genes with well-matched human homologues. *Mol Hum Reprod* 7, 521-543

Hamatani T., Falco G., Carter M. G., Akutsu H., Stagg C. A., Sharov A. A., Dudekula D. B., VanBuren V., Ko M. S. (2004) Age-associated alteration of gene expression patterns in mouse oocytes. *Hum Mol Genet* 13, 2263-2278.

These publications provided further reference points to make a shortlist of potential candidate targets not only containing the consensus sequence, but being recognised in the EST library and in addition allow classification of the identified genes into functional categories.

Furthermore, a selection of these potential targets were analysed to confirm expression within Wt and Het oocytes by RT-PCR analysis (Section 2.3) and then subjected to semi-quantitative SYBR green analysis (Section 2.4) to determine transcript expression between the Wt and Het females.

5.3. Results

5.3.1. Shortlist of candidate putative targets

After careful application of selection criteria, from the 30,000 genes present in the mouse genome, 30 were shortlisted as not only having the consensus sequence but also being expressed within the oocyte. Further criteria for selection were limited to the length of detected transcript (>24 nucleotides long) or the number of repeated potential binding sequences. It is important to note that as a direct result of the application regions which expanded beyond the sequence of selection criteria potential targets may have been missed or overlooked.

The results from this extensive *in silico* are represented in Table 5.2. Results were sorted into functional groups and the genes highlighted in green are those with transcripts longer than 24 nucleotides. Increasing transcript length may increase the probability of these being true *in vivo* targets.

Although variations in the consensus sequence were applied to the *in silico* trawl, the intention was to detect regions which expanded beyond the sequence limitation. Once the original selected genes had been subjected to an EST search there were still too many to analyse. Using the two papers reduced the number of genes further, and aided the classification of the selected genes. Potential targets included genes associated with the cell cycle, cytoskeleton, membrane receptors, signalling and communication pathways.

Table 5.2: Shortlist of *in silico* detected DAZL oocyte target mRNAs

Results from EST and Stanton and Green, (2001)

Reference and Function	Gene Name	Abbreviation		Sequence
Cell cycle				
ref|NM_007570.2|	Mus musculus B-cell translocation gene 2	Btg2		16-T3CT6GT5
ref|NM_145436.2|	Mus musculus cell division cycle 27	Cdc27		15-T4CT6CT3
ref|NM_011495.2|	Mus musculus polo-like kinase 4 (Drosophila)	Plk4		15-T2GT3GT5GT2
Cytoskeleton				
ref|NM_007615.3|	Mus musculus catenin (associated protein), delta 1	Ctnnd1		26-T7GT3GT3GT4GT5 10-T3GT3CT2
ref|NM_008943.2|	Mus musculus presenilin 1	Psen1		24-T4GT4CT3G
Secretory pathways, exocytosis, endocytosis ect				
ref|NM_007479.3|	Mus musculus ADP-ribosylation factor 4	Arf4		10-T2GT4GT2
Membrane receptors, ion channels ect				
NM_007664.2|	Mus musculus cadherin 2	Cdh2		19-T4CT5GT3GT3C
ref|NM_001080814.1|	Mus musculus FAT tumor suppressor homolog 3	Fat3		10-T3CT3CT2 10-T2CT4CT2
ref|XM_885736.2|	Mus musculus fat tumor suppressor homolog	Fath		22-T2GT4GT7CT5G
ref|NM_008943.2|	Mus musculus presenilin 1	Psen1		24-T4GT4CT3G
ref|NM_009194.2|	Mus musculus solute carrier family 12, member 2	Slc12a2		20-T6CT5CT7
Structural nuclear proteins				
NM_010880.3|	Mus musculus nucleolin	Ncl		26-GT7GT6GT3GT4GT
NM_183392.2|	Mus musculus nucleoporin 54	Nup54		
Phosphatases				

NM_011200.1|	Mus musculus protein tyrosine phosphatase 4a1	Ptp4a1		11-GT2GT6G
Protein degradation				
ref|NM_011192.3|	Mus musculus proteaseome (prosome, macropain) 28, 3	Psme3		16-T5GT6CT2C
ref|NM_198091.2|	Mus musculus ubiquitin specific peptidase 2	Usp2		23-T6CT4GT7CT2C
Signalling pathways				
ref|NM_172695.2|	Mus musculus phospholipase A2, activating protein	Plaa		14-T4GT5CT3
ref|NM_009535.2|	Mus musculus Yamaguchi sarcoma viral (v-yes) oncogene homolog 1	Yes1		14-T3GT5CT4
RNA transcription				
NM_010613.2|	Mus musculus KH-type splicing regulatory protein	Khsrp		67-TCTCT7CT10CT5CT6CT6CT5CT18
ref|NM_145457.3|	Mus musculus polyadenylate binding protein-interacting protein 1, transcript variant 1, 2	Paip1		13-T2GT3GT3GT2 19-T3CT5GT2GT5C 11-T3GT3CT2C

In paper but not identified as EST

ref|NM_009829.3|	Mus musculus cyclin D2	Ccnd2		34-T4GT4GT9GT13C
ref|NM_011992.2|	Mus musculus reticulocalbin 2	Rcn2		19-T5GT8GT4

Results from EST and Hamatani et al., (2004)

Cell communication				
NM_007664.2|	Mus musculus cadherin 2	Cdh2		19-T4CT5GT3GT3C
Ubiquuitine proteasome pathway				
ref|NM_016786.3|	Mus musculus huntingtin interacting protein 2	Hip2		42-T2CT7GT6GT7GT7GT7G
ref|NM_009174.3|	Mus musculus seven in absentia 2	Siah2		20-T5GT2GT5GT5

Mitotic cell division, microtubule cytoskeleton, ATP-binding				
ref|NM_001081453.1|	Mus musculus ninein, transcript variant 1	**Nin**		**17-T5CT5CT4C**
Guanyl nucleotide binding, cell signaling				
ref|NM_001081105.1|	Mus musculus ras homolog gene family, member H	**Rhoh**		**24-T4CT9CT9**
Chromosome organization and biogenesis, ATP-binding				
ref|NM_172937.3|	Mus musculus SNF2 histone linker PHD RING helicase, transcript variant 2	**Shprh**		**14-T3CT4CT2CT2**
Regulation of transcription				
ref|NM_001080132.1|	Mus musculus thymopoietin, transcript variant 2	**Tmpo**		**14-T5GT3GT4**

Not positive for EST

ref|NM_194268.2|	Mus musculus one cut domain, family member 2	**Onecut2**		**13-T4CT4GT3**
NM_177386.3|	Mus musculus Scm-like with four mbt domains 2	**Sfmbt2**		**52-GT6GT3GT6[GT4]4GT7GT3GT**
ref|NM_011462.2|	Mus musculus spindlin , transcript variant 1	**Spin1**		**28-T2GT10CT14**

Similar between paper and positive EST		**Hit**	**Paper**	
ref|NM_007479.3|	Mus musculus ADP-ribosylation factor 4	**Arf4**	**Arf1**	**10-T2GT4GT2**
ref|NM_019835.2|	Mus musculus UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	**B4galt5**	**B4galt6**	**29-T2GT6CT2GTGT4GT2CT5G**
ref|NM_033370.3|	Mus musculus coatomer protein complex, beta 1	**Copb1**	**Copb2**	**11-T3CT3GT2C**
ref|NM_145457.3|	Mus musculus polyadenylate binding protein-interacting protein 1 transcript variant 1	**Paip1**	**Paip2**	**13-T2GT3GT3GT2**

5.3.2. PCR analysis of selected targets

From the shortlist of potential oocyte DAZL target mRNAs four genes were further selected for subsequent analysis comparing Wt and Het oocytes to determine if functional gene number is affecting target mRNAs. The four genes selected for further analysis were Cdh2, Ctnnd1, Khsrp and Paip. The justification for this gene selection relates to their potential function within the oocyte which may lead to the observed differenced noted between the Wt and Het litter sizes. In particular Cadherins (Cdh2) and catenins (Ctnnd1) have been are associated with cellular communication, forming junctions between cells and are essential for cell proliferation all possible mechanisms which may be targeted and contribute to the differences between Wt and Het phenotype (Stockinger et al., 2001). Furthermore Khsrp was selected due to the presence of an extensive DAZl binding region increasing the probability of it being a potential target and Paip which is a Poly A binding proteins, which in turn have been previously shown to be associated with DAZL proteins (Gray et al., 2000). All four selected genes were present in mRNA from d10 and d21 Wt and Het ovaries in addition to oocyte pools (Figure 5.2). However they were also present in the KO ovary, suggesting that although these genes have been shown to be expressed in the oocyte, they are not oocyte specific.

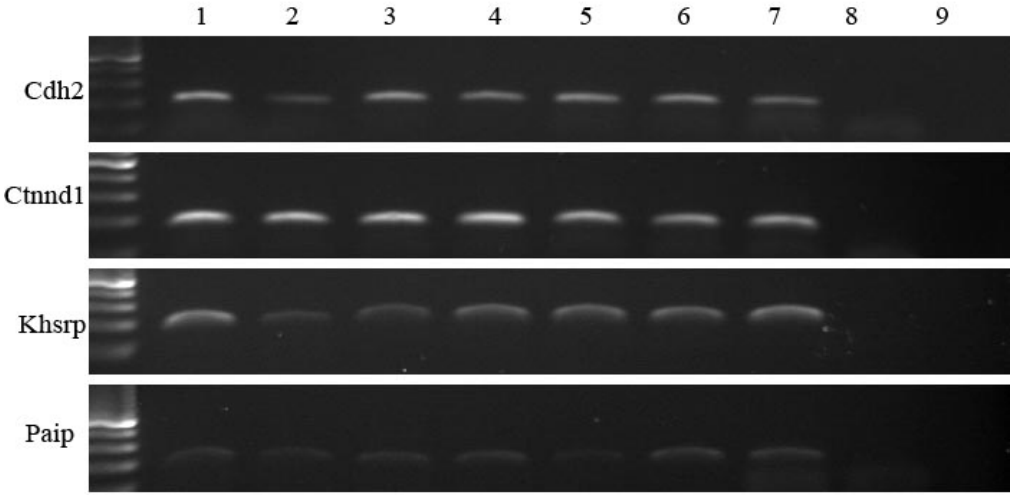

Figure 5.2: PCR screen of selected target genes within the developing ovary d21 (1) Wt and (2) Het, d10 (3) Wt and (4) Het and oocyte pools (5) Wt and (6) Het and (7) d21 KO ovary was also used. Rt-ve (8) control and (9) water control.

Following the initial RT-PCR analysis these genes of interest were subjected to real-time quantitative PCR (Figure 5.4). As a consequence of the low expression of Paip transcripts the results from the real time PCR could not be quantified as the detection range was beyond that of the threshold levels. Wt and Het d21 oocyte pools were used to determine if the expression of two functional copies (Wt) compared to one functional copy (Het) had any effect in an attempt to identify oocyte DAZL targets. Results from the real-time quantitative PCR demonstrated that Cdh2 and Ctnnd1 mRNA expression was not significantly different between the two genotypes. Khsrp mRNA expression was significantly ($P<0.05$) up-regulated in the Het compared to the Wt suggesting an increase in functional translation in the Het oocytes (Figure 5.3).

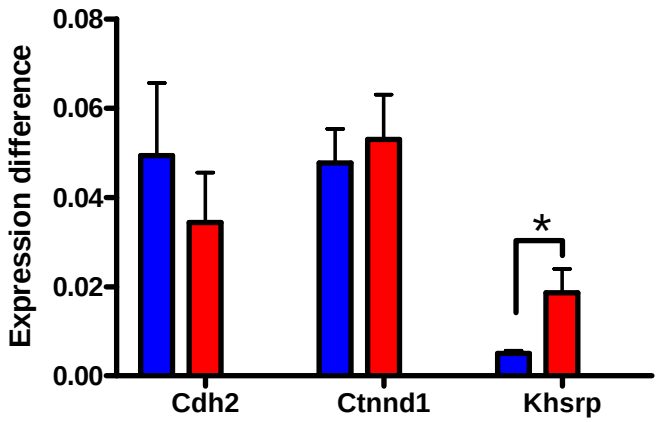

Figure 5.3: mRNA expression of Cdh2, Ctnnd1 and Khsrp putative DAZL targets. No difference in expression was observed between the Wt and Het for Cdh2 and Ctnnd2, significant difference in mRNA expression between Wt and Het for Khsrp * $P<0.05$ Wt and Het .

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5.4. Discussion

Identifying RNA targets of known RNA-binding proteins has proven more challenging than identifying proteins that bind to known RNAs and this is also true for identifying DAZL targets (Venables et al., 2001). A complementary approach has been utilised within this chapter to identify RNA targets of mouse oocyte DAZL in order to gain insight into its mode(s) of action. As repeatedly mentioned not only is DAZL essential for oogenesis, the phenomenon observed in the Hets also needs to be addressed, whereby fertility is augmented. One hypothesis is that whichever RNAs are regulated by DAZL are in turn responsible or partly responsible for the increase in fertility observed in the Het mice in which only a single functional copy of DAZL is present.

The main focus of this current chapter was to use an alternative Bioinformatics approach, which can be described as "the application of computational methods to biological problems", with our biological problem being the identification of DAZL targets. Furthermore, bioinformatics is a widely applied interdisciplinary research tool which uses the sequence, structure and functions of genes and genomes along with the corresponding products and uses the available technology for storage, retrieval, manipulation and distribution. In the current investigations we have identified and employed the use of the pre-published consensus sequence (Venables et al., 2002) and NCBI software to identify *in silico* DAZL targets. It is important to note that bioinformatic predictions are not formal proof of biological concepts or functions, and, although complimenting experimental biology they do not replace traditional experimental research methods for testing functional biological hypotheses (Kaminski, 2000; Goodman, 2002).

From the bioinformatic methodologies used in this current chapter, the potential target Khsrp was identified as having an extensive length of DAZL consensus sequence, in addition to being identified in oocyte EST libraries and in the papers of interest. Furthermore, Khsrp did show a significant increase in transcript expression in Het oocyte samples compared to that of the Wt. Khsrp itself has KH domains which directly mediate RNA binding, mRNA decay and interactions with the

exosome and poly (A) ribonuclease (Gherzi et al., 2004), all function which are applicable to the oocyte. It can now be proposed that in addition to the published targets and suggested functions DAZL may be involved in binding complexes with Khsrp and subsequently involved in mRNA decay. With the unsuccessful attempt in apply the UV CLIP method (Chapter 4), we are unable to confirm *in vivo* interactions of selected mRNA targets and subsequent approaches may now need to be addressed.

Two additional candidate target genes were selected and analysed using real-time PCR, cadherin-2 (Cdh2) and catenin-1 (Ctnnd1). Both genes were identified to be expressing the consensus sequence but no significant difference in mRNA expression was identified between the two genotypes. Cadherin-2 also known as N-cadherin is associated with catenins and involved in cell to cell communication. Interestingly these complexes have many functions associated with cell adhesion and migration and blood vessel morphogenesis all of which may be applied to the oocyte and granulosa cells. Furthermore, N-cadherin can be cleaved by presenilin-1 (Marambaud et al., 2003) which also has the DAZL consensus sequence although this was not investigated further. These two associated genes of interest cannot be totally ruled out as DAZL targets in spite of there being no alteration in expression between the Wt and Het, but once again any interactions have to be confirmed *in vivo*.

One systematic problem with the application of bioinformatics in an attempt to identify individual candidate targets was a substantial number of genes exhibited the selected consensus sequence. In addition, the selection criteria used may have limited and masked true oocyte DAZL targets or it is possibly that the specificity of the consensuses sequence used (Venables et al., 2001) is too ambiguous to be applied in this way. Therefore, bioinformatics should not be used alone and should only be used to aid biological experimentation or at least provide guidance in the right direction. It may be that the alternative sequence (Jiao et al., 2002) may provide additional DAZL targets, but once again until *in vivo* techniques have been established in the oocyte any potential targets cannot be confirmed.

To this end we have shown the promise of using an *in silico* bioinformatics approach to reveal potential mRNAs which may bind to DAZL as a result of the presence of consensus target sequence. EST libraries in conjunction with published oocyte genes have been used to facilitate the further selection of potential targets. Until now no direct targets have been determined *in vivo* for DAZL function within the oocyte. It would now be ideal if the techniques applied in Chapter 4 could be utilised in conjunction with the results from this chapter to confirm the possible interactions between DAZL and these identified oocyte target genes. Many more DAZL mRNA target candidates could have been selected for further analysis, and these now require the application of functional methodologies that can be applied biologically to verify direct interactions and functional outcomes.

Chapter 6: Ovarian role of putative DAZL target PDCD4

6.1. Introduction

The methods used in Chapter 5, whereby the DAZL consensus sequence (Venables et al., 2001) was employed to identify DAZL targets within the mouse genome had been previously exploited to identify potential DAZL candidates within the human genome (Ismail, MSc 2007). Once again a high number of potential targets were identified and by process of elimination by ovarian specificity and potential function, the candidate gene identified and selected for further investigation was programmed cell death factor 4 (PDCD4). PDCD4 was not originally identified in the mouse bioinformatics trawl, but on investigation into the expression of the consensus sequence within the mouse gene homologue it was revealed that a DAZL binding motif was located at the 3'mRNA of this potential target (Figure 6.1). Furthermore, with the understanding that DAZL affects litter size by a mechanism unknown, a potential function for PDCD4 in programmed cell death within the developing follicle resulting in an increase in ovulation and decrease in atresia in the Het females led to further investigation of this potential target.

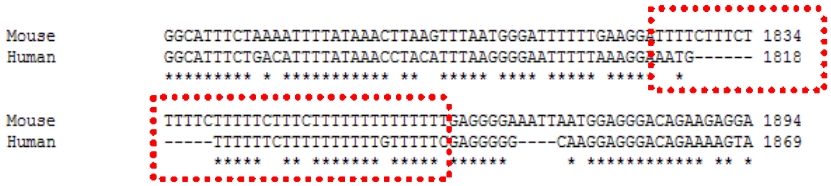

Figure 6.1: Human and mouse PDCD4 mRNA sequences exposing potential DAZL binding motifs present within the 3' UTR.

PDCD4 is also known as MA3 (Shibahara et al., 1995), TIS (Onishi et al., 1998) 197/15a (Azzoni et al., 1998) H731 (Cmarik et al., 1999) and DUG (Goke et al., 2002) and was originally isolated as a gene activated in cells during apoptosis and commonly up-regulated upon induction of cell death (Shibahara et al., 1995). Furthermore, PDCD4 has been identified as a translation factor which to bind to

eIF4A (Goke et al., 2002; Yang et al., 2003; 2004), inhibiting protein translation and expression is strongly associated with a decline in tumour progression. Latterly, PDCD4 has been briefly reported to be expressed abundantly in oocytes and in embryos through the two-cell stage (Jurisicova et al., 1998), but how and when it functions within the steroidogenically active ovary is unknown. In addition it has been reported that PDCD4 deficient mice which express many detrimental phenotypic characteristics (spontaneous lymphomas, significantly reduced life span, altered oncogenesis and inflammation) also develop multi-organ cysts which involved the ovary in 50% of animals (Hilliard et al., 2006). Furthermore, PDCD4 expression has been shown to be down-regulated in ovarian tumors (Bonome et al., 2005). Interestingly, unilateral ovarian hyperplasia and cystic tumour formation are also characteristic features of the DAZL KO ovary (McNeilly et al., SRF 2008) which might suggest that the lack of DAZL may be linked to a decrease in expression of PDCD4 controlled apoptotic control hence tumour growth. Thus, PDCD4 may be involved as a positive regulator in programmed cell death within the ovary or indeed as a functional component of the transcription mechanism involving DAZL and eIF4A. These potential connections were the major motivating factors for the studies in this chapter and in order to investigate the possibility that PDCD4 plays an active role associated with DAZL and in particular regulating programmed cell death, it was necessary to expand and clarify the understanding on PDCD4 expression and localisation within the ovary. These parameters were therefore investigated first and related to potential function, again recognising that the functional copy number of DAZL in the Het phenotype may be having interacting effects.

During these investigations of the potential role of PDCD4 as a DAZL target it became apparent that this cell death associated protein was abundantly expressed within the developing ovary, and of particular interest, was the difference in nuclear to cytoplasmic localisation within the corpus luteum. Further to these findings, induced follicle atresia, natural apoptosis associated with pro-oestrous, and interruption to pregnancy through the use of luteolytic mediators bromocriptine and cloprostenol were also investigated to determine potential functional roles of PDCD4

within the ovary. Having an understanding of the cellular and molecular mechanisms that activate and execute programmed cell death in the female germ line has implications for therapeutic management as does understanding the importance of DAZL expression within oocyte development.

6.2. Materials and Methods

6.2.1. Identification of PDCD4

In brief, by the same *in silico* approached used in Chapter 5 (Section 5.2) the human genome was subjected to a BLAST search (NB: the initial BLAST search was performed by Dr Wail Ismail with all subsequent analysis and investigations performed by myself) using the different permutations of the DAZL binding motif (Venables et al., 2001) to identify potential putative RNA targets to which DAZL may bind and regulate mRNA expression. As mentioned a potential DAZL binding site is located in the murine PDCD4 sequence, however investigation of the patterns of expression and elucidation of potential function were required. To verify PDCD4 expression within Wt and Het oocytes (d21) and Wt and Het ovarian tissues (d21 and d10 mice) specific oligonucleotide primer sets were designed for mouse PDCD4 and used to detect reverse transcription products by conventional PCR (Section 2.3.4). In addition real-time light cycler SYBR green PCR was performed to detect differences in PDCD4 transcript number between the two genotypes (Section 2.4). The protein expression of PDCD4 was detected using immunohistochemistry, performed using the standard protocol (Section 2.8).

6.2.2. Role of PDCD4 in ovarian apoptosis

To investigate the potential roles of PDCD4 as a putative DAZL target the genes, in additions to its recognised pro-apoptotic function, studies were designed using mice to observe natural atresia in follicles and corpora lutea, induced follicle atresia, and disrupted CL function through induced luteolysis.

6.2.2.1.Natural ovarian apoptosis

Pro-oestrus animals were used to investigate natural atresia associated with corpus luteum degeneration during the oestrous cycle, whereby old luteinized corpora lutea undergoing functional regression should be present. Cycling adult virgin mice were smeared daily (Judy McNeilly) to determine the stage of the oestrous cycle. The sample vaginal smears were analysed under a microscope to determine the cytology of the cells present, hence determine cycle stage. Animals were selected depending on the stage of cycle for ovarian collection and further analysis.

6.2.2.2.Induced apoptosis: modulation by FSH withdrawal

In order to investigate apoptosis within developing follicles a regime was designed whereby folliculogenesis was induced by the administration of recombinant human FSH (rech FSH) to pre-pubertal mice (d21) followed by FSH withdrawal. The withdrawal of FSH from the system was achieved by neutralising the exogenous rech FSH by treatment with a rabbit anti-human FSH antibody (M91: 100µl ip) and endogenous FSH secretion was also inhibited by treatment with ovine follicular fluid (oFF; 100µl ip: Crawford et al., 2004) as a source of inhibin (Figure 6.2). The antibody against FSH should immuno-neutralise the circulating recombinant FSH whereas the oFF inhibits secretion of any endogenous FSH present through a direct action on the pituitary (Brown et al., 2001). This regime of FSH stimulation followed by acute withdrawal was hypothesised to induce follicle atresia. Four animals were used in each treatment group.

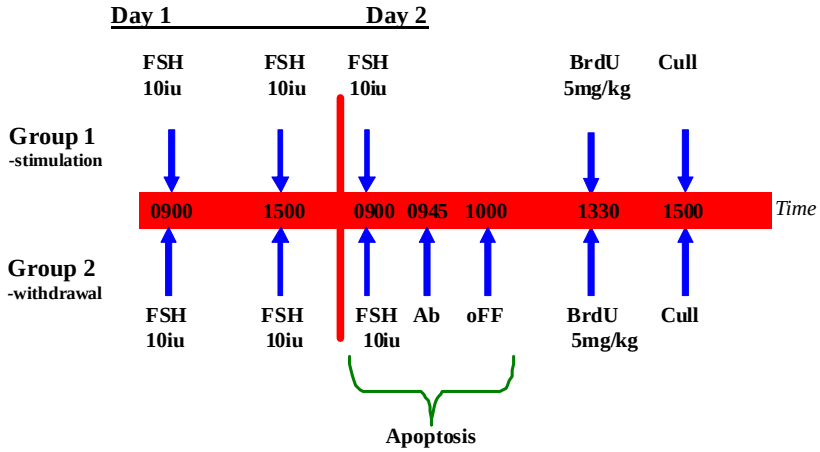

Figure 6.2: Schematic summary of FSH treatment regime applied to d21 pre-pubertal mice to induced folliculogenesis (Group 1; n=4) and induced apoptosis (Group 2; n=4) by treatment and withdrawal of FSH.

6.2.2.3.Chemically induced luteolysis

Luteolysis is the natural structural and functional degradation of the CL. However it can be chemically induced by direct and indirect methods. Bromocriptine and cloprostenol were selected as luteolytic agents to investigate cell death within the CL and in understanding their disruptive mechanism to induce cell death it may aid the identification of potential roles of PDCD4 protein within the ovary. Bromocriptine is a chemical dopamine agonist which inhibits prolactin synthesis and secretion by directly binding to dopamine D2 receptors on the pituitary lactotrophs (Vance et al., 1984). A decrease in prolactin results in a reduction of progesterone and hence induces luteolysis within the CL of the pregnant rodent (Zetser et al., 2001). Cloprostenol is a functional analogue of prostaglandin F2α having a specific luteolytic action at the ovarian level. It causes functional and morphological regression of the CL in ungulates, marmosets and rodents (Nancarrow et al., 1982; Summers et al., 1985; Torjesen and Aakvaag, 1986) followed by a return to oestrous and normal ovulation. The functional hypothalamic-pituitary-ovarian axis and the disruptions caused by bromocriptine and cloprostenol are summarised in Figure 6.3.

Female mice caged with a stud male were checked daily for the appearance of vaginal plugs indicating mating and potential conception. On the seventh day of gestation the females were injected intra peritoneal (ip) with bromocriptine (4mg/kg; 120μg/30g mouse, Sigma) (2-bromo-α-ergocrytine.methanesulfonate salt B2134-25MG 095K1528) or with cloprosetenol ((Estrumate synthetic Prostaglandin Schering-Plough Animal Health) dose 5μg/mouse)). Sixteen hours after the luteolytic treatments, the animals were given injections of BrdU, (5mg/kg ip) and subsequently the mice were euthanized after an additional two hours. Body weight was recorded, blood was collected by cardiac puncture and the ovaries were removed and weighed. The number of visible corpora lutea were counted, the condition of the uterus noted and the number of implantation sites were recorded. Ovaries were fixed in NBF (Section 2.7), for subsequent analysis and blood collected for LH and FSH radioimmunoassay (Section 2.9, performed by Judy McNeilly).

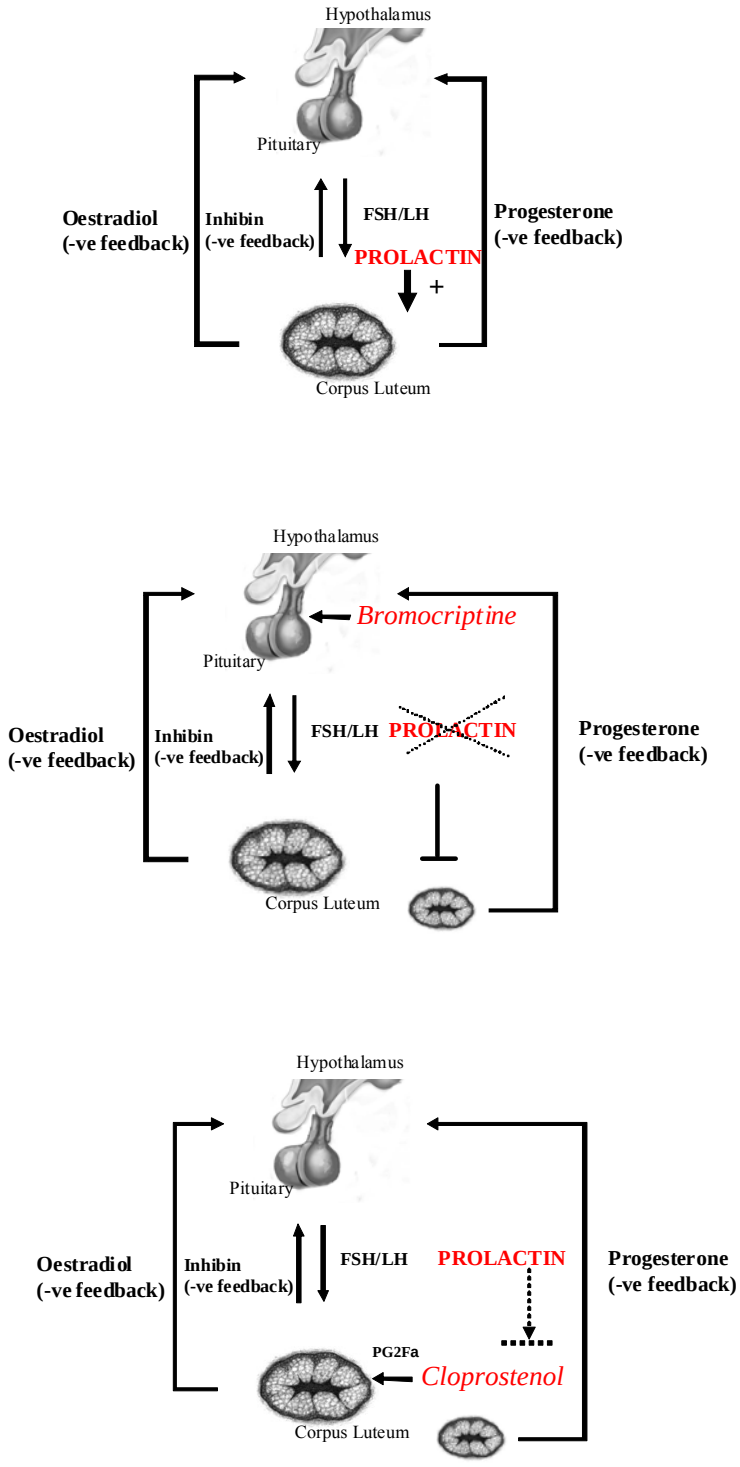

Figure 6.3: Summary of hypothalamic-pituitary-ovarian interaction during the luteal phase: actions of bromocriptine and cloprostenol on prolactin function and luteolysis.

6.2.3. Analysis

To assess the degree of induced atresia, whether natural, by FSH withdrawal or by inducement of luteolysis, ovaries from the different treatment groups were sectioned and immunostained for markers of proliferation (PCNA or BrdU (Section 2.8)) and the apoptotic marker cleaved caspase 3, in addition to PDCD4, the protein of interest. Observations were made on the relationship between PDCD4 localisation and expression compared to that of cleaved caspase 3 expression. Visual analysis of cleaved caspase 3 expression was performed from the ovaries which had been subjected to FSH stimulation/withdrawal. In addition mRNA expression of PDCD4 and cleaved caspase 3 was analysed using SYBR green RT-PCR (Section 2.4.2). The number of corpora luteum were recorded in the induced luteloysis groups in addition to the appearance of the nuclear compared to cytoplasmic localised expression of PDCD4 in comparison to the pregnant controls.

6.3. Results

6.3.1. PDCD4 as a potential DAZL target

It is assumed that the functional copy number of DAZL would potentially determine the transcription levels of functional DAZL targets. Therefore the expression of the recently identified putative target PDCD4 was firstly compared between the DAZL Wt and Het females. PDCD4 mRNA expression levels were investigated in d21 oocyte pools (localised DAZL expression) and d10 ovaries (uniform population of same stage oocytes) as previously used in Chapter 3. The expression levels of PDCD4 were unchanged between the Wt and Het samples at both d21 (oocyte pools) and d10 (ovaries) (Figure 6.4).

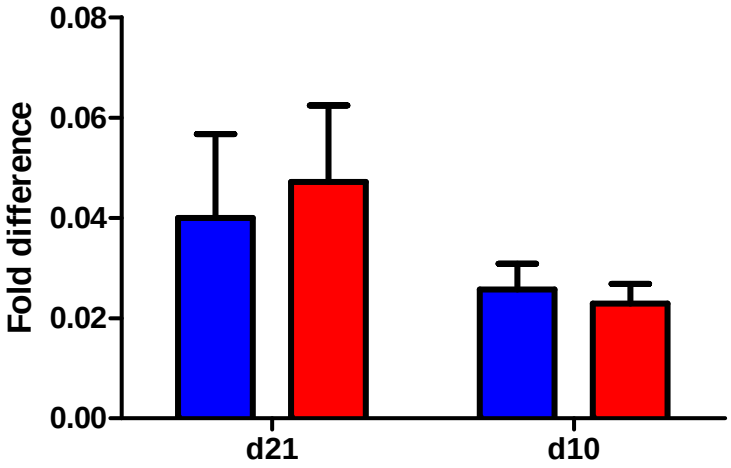

Figure 6.4: Quantitative analysis of PDCD4 mRNA fold difference between Wt and Het in d21 oocyte samples (n=5) and d10 ovaries (n=5). Values are means ± SEM (Wt and Het).

6.3.2. Novel expression of PDCD4 in the mouse ovary

Having confirmed that PDCD4 mRNA was present in the mouse ovary, and although no differences in mRNA expression were detected between the Wt and Het, we then established the pattern of PDCD4 protein expression within ovarian tissue using immunohistochemistry. Initially d21 ovary, adult ovary with corpus luteum present, oviduct, DAZL KO d21 ovary and tumor samples from a DAZL KO ovary were analysed to enable a more informed interpretation of PDCD4 expression within the female reproductive tract. Review of PDCD4 protein immunoexpression revealed positive cytoplasmic localisation within the oocytes of the d21 ovary, both in the

small primordial oocytes and oocytes within the larger pre/antral follicles (Figure 6.5 A). In addition PDCD4 was located to the granulosa cell cytoplasm, with light immunostaining present in the surrounding thecal layers (Figure 6.5 A). PDCD4 protein expression was additionally localised to the ovarian surface epithelium (OSE) surrounding the ovary where expression was strong (Figure 6.5 B) and to the steroidogenic cell of the corpus luteum (Figure 6.5 B). Within the corpus luteum PDCD4 was expressed in both the cytoplasm and in the nucleus, which prompted further investigation (Section 6.3.4 and 6.3.5). Furthermore, the oviduct epithelial cells intensely expressed PDCD4 protein (Figure 6.5 C) as did the OSE and cells within the KO ovary (Figure 6.5 D). In the KO ovarian tumor and consistent with previous reports illuminating the decrease in PDCD4 expression associated with tumor progression, PDCD4 immunoexpression appeared reduced in the ovarian tumor sample (Figure 6.5 E).

The observations of PDCD4 expression and the striking nuclear localization in cells within the CL compared to cytoplasmic expression in the granulosa cells of follicles suggested possible translocation of this protein with regards to its active function. These observations subsequently contributed to the series of investigations addressing natural and induced apoptotic functions within the ovary to identify potential function of PDCD4 as a pro-apoptotic factor.

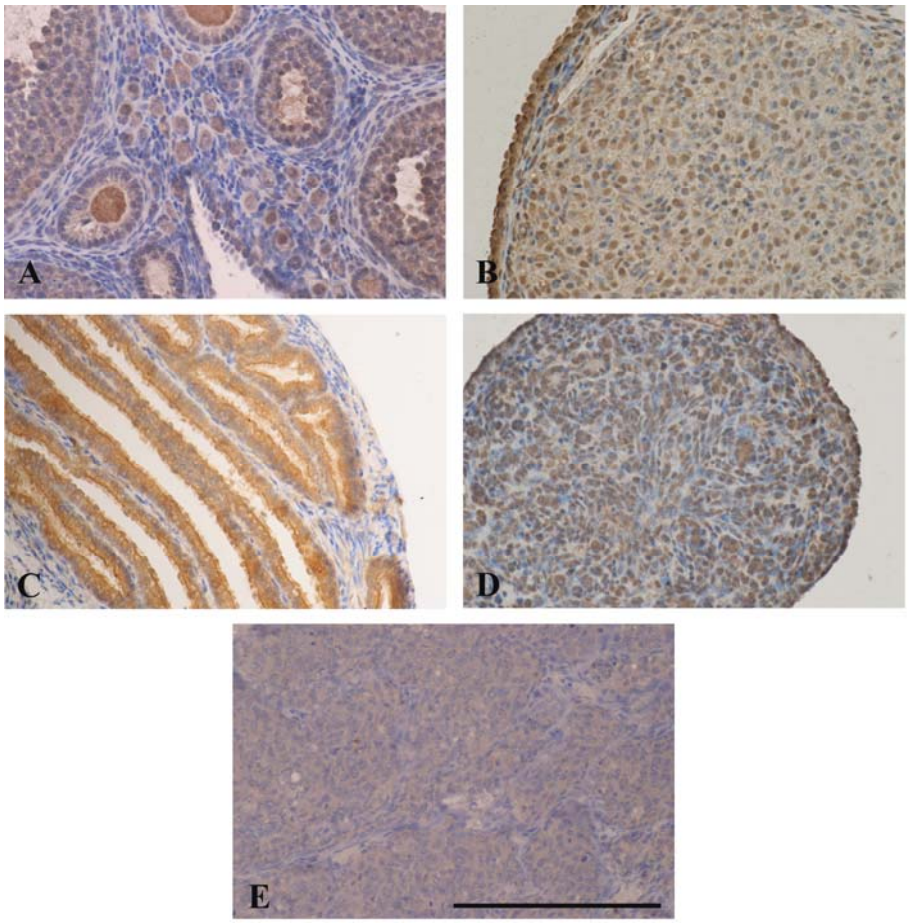

Figure 6.5: Immunoexpression of PCDC4 in representative sections of d21 ovary (A), adult ovary corpus luteum (B), oviduct (C), d21 DAZL KO ovary (D) and a tumor from a DAZL KO ovary. Scale bar represents 100μm.

6.3.3. PDCD4 mRNA and protein expression during follicle apoptosis

Evaluation of cell proliferation markers, PCNA and BrdU were investigated using immunohistochemistry after FSH stimulation or after FSH withdrawal of d21 ovaries, to enable the detection of proliferating cells (Figure 6.6). PCNA is a cell cycle protein which holds DNA polymerase delta to DNA with expression increasing during the G1-phase, peaking at the S-phase, and declining during the G2/M-phases of the cell cycle (Foley et al., 1993). PCNA was detected within granulosa cells and stromal cells (Figure 6.6 A and B) of the two treatment groups. As a result of the prolonged expression through the cell cycle no obvious differences were observed

between the two treatment groups, although cells did show different degrees of staining intensity which one would expect. Furthermore, BrdU is present only the S phase of the cell cycle within which it is incorporated into newly synthesized DNA of replicating cells, substituting thymidine during replication. In comparing the subsequent sections (Figure 6.6 C and 6.6 D), large atretic follicles have a substantial reduction in BrdU protein expression compared to the surrounding proliferating follicles.

In comparison to these intrinsic proliferative markers, cleaved caspase 3 was used to identify apoptotic regions within the ovary and used as a comparative marker for the expression of the pro-apoptotic PDCD4. An increase in cleaved caspase 3 expression was notably observed after the FSH withdrawal treatment, clearly indicating follicle atresia (Figure 6.6 E). Both an increase in pyknotic nuclei and an increase in cytoplasmic cell degradation was observed. This increased cleaved caspase 3 expression was not observed in the FSH stimulation group (Figure 6.6 F). Interestingly even in the classified atretic follicles identified by the expression of cleaved caspase 3 and pyknotic nuclei, cell proliferation was also observed with reference to BrdU and PCNA positive immunoexpression, although expression of BrdU was reduced. There was no identifiable difference in the localization or intensity of PDCD4 protein between the two FSH treatment groups. If PDCD4 is acting as a pro-apoptotic factor in follicles one would presume either a decrease in expression at the onset of induced follicle atresia or a functional translocation to activate its activity within the apoptotic cell of the follicle.

In order to further investigate the possibility that PDCD4 may play a role in ovarian apoptosis, ovaries from the d21 animals subjected to the FSH stimulation and withdrawal were additionally used to analyse cleaved caspase 3 and PDCD4 mRNA expression. Interestingly the expression of cleaved caspase 3 was reduced after the FSH withdrawal treatment compared to FSH stimulation treatment, in contrast to the apparent increase in protein expression. However, cleaved caspase 3 mRNA may have become degraded as a result of apoptosis despite protein remaining. In support

of the absence of change in the expression of PDCD4 protein, no difference in PDCD4 mRNA expression was observed between the two treatment groups (Figure 6.7), further suggesting that PDCD4 is not involved in induced follicle atresia regardless of its association as a pro-apoptotic factor.

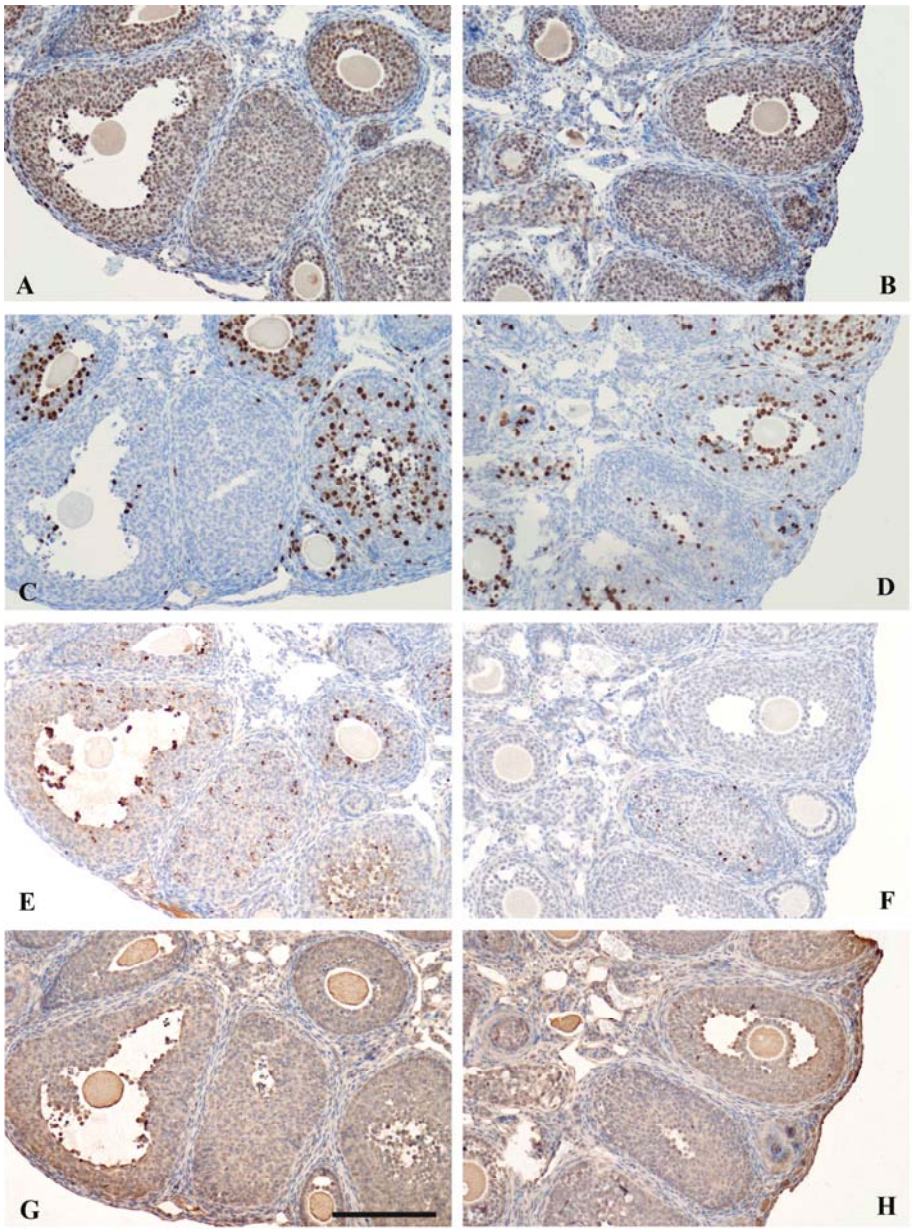

Figure 6.6: Immunoexpression of PCNA (A: FSH withdrawal and B: FSH stimulation), BrdU (C: FSH withdrawal and D: FSH stimulation), Cleaved caspase 3 (E: FSH withdrawal and F: FSH stimulation) and PCDC4 (G: FSH withdrawal and H: FSH stimulation). Scale bar represents 100µm.

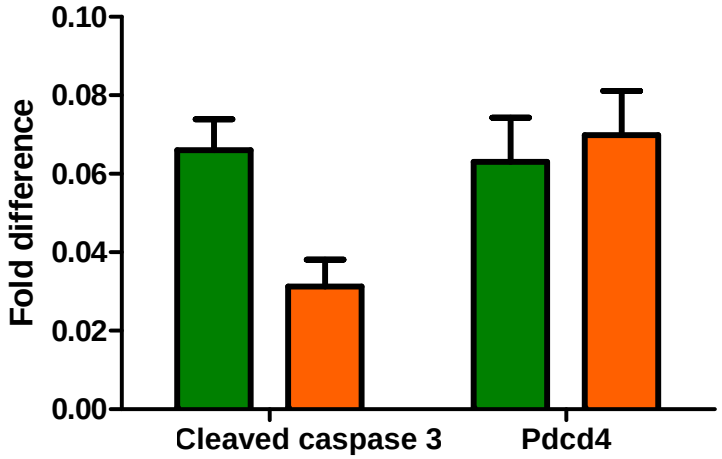

Figure 6.7: Quantitative analysis of cleaved caspase 3 and PDCD4 mRNA levels after FSH stimulated compared to FSH withdrawal treatment d21 ovaries (n=4). Values are means ± SEM.

6.3.4. PDCD4 protein expression during natural apoptosis

In order to investigate a process of natural apoptosis within the ovary and in addition to the PDCD4 protein localisation identified within the CL, pro-estrous animals were screened again for the two proliferative markers PCNA and BrdU (Figure 6.8 A-D). Representative images from two different animals confirmed that there is extensive proliferation occurring in the pro-estrous ovary, with a reduced expression of PCNA within the corpora luteum and only a few BrdU positive cells. Apoptotic follicles were observed within the pro-estrous ovaries, and as indicated by cleaved caspase 3 protein expression, very few cells within the CL are apoptotic (Figure 6.8 E-F). Furthermore, the expression of PDCD4 notably changed within the CLs examined, ranging from light cytoplasmic expression with limited nuclear expression (Figure 6.8 G) to more intensely expression and localised to the nucleus in the steroidogenic cells (Figure 6.8 H). These differences observed in CL PDCD4 immunoexpression, visually correlated to the expression of cleaved caspase 3, whereby, when PDCD4 was light with reduced nuclear expression a higher number of positive cleaved caspase 3 cells were present with the CL. Conversely, when nuclear PDCD4 expression was observed in the CL, little or no cleaved caspase 3 was identified.

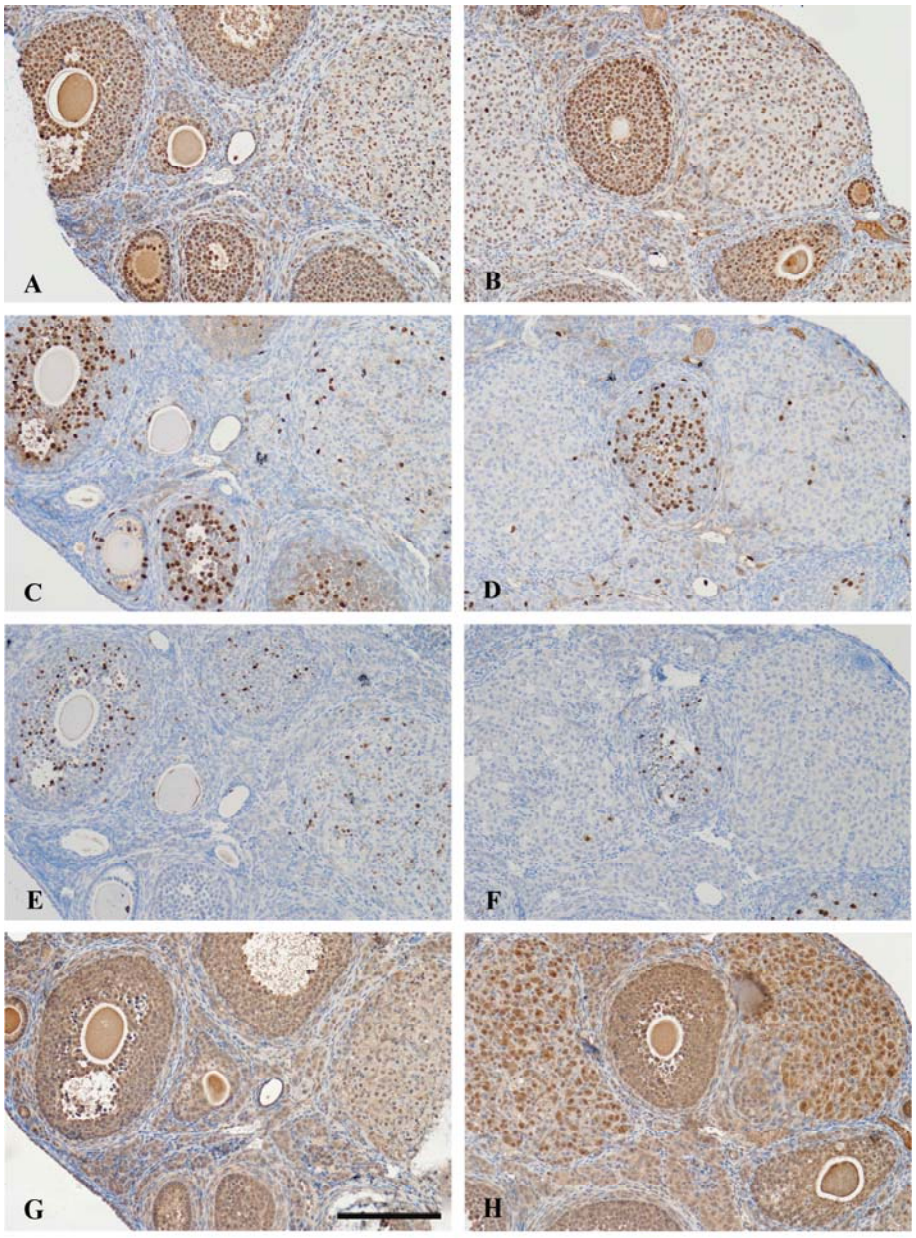

Figure 6.8: Representative photomicrographs depicting immunoexpression of proliferation markers PCNA (A and B), BrdU (C and D), apoptotic marker Cleaved caspase 3 (E and F) and protein under investigation PCDC4 (G and H) in pro-estrous animals. Scale bar represents 100µm.

6.3.5. PDCD4 protein expression in chemically induced luteolysis

Exposure to bromocriptine and cloprostenol induced functional luteolysis and disruption of pregnancy compared to the non-treated pregnant controls. Variation in the effects on the uterus, implantation sites and ovary were recorded (Table 6.1).

Table 6.1: Ovarian and uterine observations of chemically induced luteolysis.

Treatment	Day of pregnancy	Observation
Bromocriptine	8	Uterus red and swollen
	8	Very swollen
	9	Swollen enlarged uterus
	8	8 visible implantation sites
Cloprostenol	6	10 visible implantation sites
	6	6 visible implantation sites
	7	11 visible implantation sites
	8	White ovaries, implantations but not healthy
	9	White ovaries, implantations but not healthy
Control	8	11 visible implantation sites
	7	9 visible implantation sites
	8	12 visible implantation sites

Changes in CLs were clearly visible in the different treatment groups when compared to the non-treated control, with the result that abnormally unorganized areas of cells were present. The structural analysis of the CL suggest that upon chemically-induced luteolyis, CL degradation and structural regression is accompanied with an infiltration of epithelial type cells, which do not express PDCD4 protein (Figure 6.9 C, D and F). The degree of abnormally organized cells and infiltration of epithelial type cells varied substantially. It was clearly evident that the CL present in the non-treated pregnant controls (Figure 6.8 G and H) were more uniform in structure presenting with healthy round steroidogenic cells in contrast to the treated groups showing luteolyic changes in the CLs. In addition the control animal showed a more overall cytoplasmic expression of PDCD4 in comparison to PDCD4 expression being nuclear and cytoplasmic in the treated animals. Furthermore, overall expression of PDCD4 was higher in the treated animals, thus

suggesting that the intensity and nuclear localization within the CL is linked to the morphological changes and structural regression as a result of disruption of prolactin function. Although not shown there was very little cleaved caspase expression present in the CLs analysed from both the treatments inducing luteolysis, with the majoring of CLs remaining negative. The highest proportion of caspase 3 expressing CL was from the bromocriptine treatment groups, suggesting that the actions of the two luteolytic factor are possibly inducing regression at a different rate. Differences were also notable between the pregnant CLs and the pro-estrous CLs (Figure 6.9 A and B (pregnant) and G and H (pro-estrous)), with the pro-estrous CLs expressing a higher degree of nuclear expression compared to the mainly cytoplasmic expression of PDCD4 in the pregnant CLs.

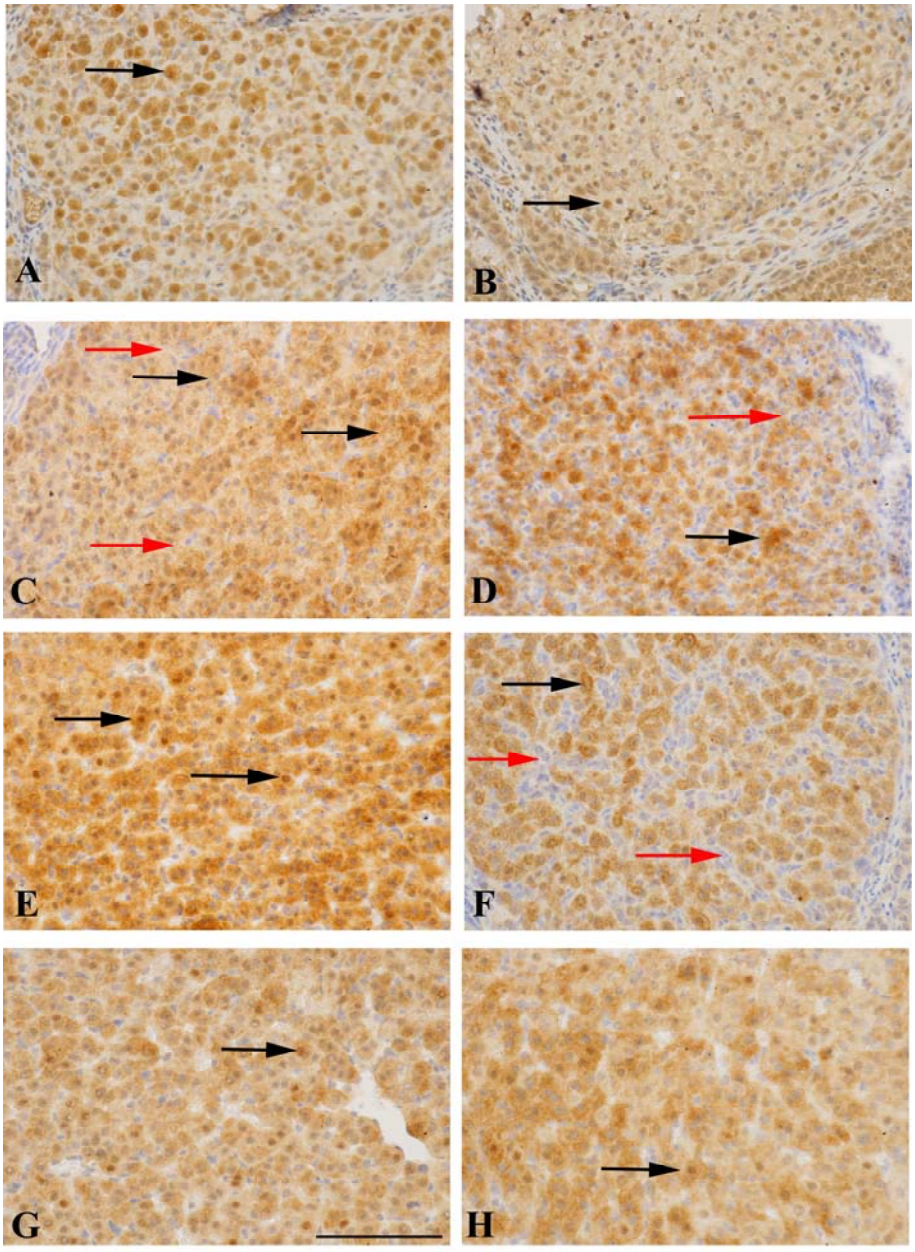

Figure 6.9: Representative photomicrographs depicting immunoexpression of PDCD4 in CLs of pro-estrous animals (A and B), bromocriptine (C and D), cloprostenol (E and F) and the non-treated pregnant control (G and H). Black arrows indicate representative areas with nuclear localization, red arrows indicate representative areas of epithelial cell infiltration. Scale bar represents 100µm.

The plasma levels of the gonadotrophins LH and FSH were measured after exposure to the luteolytic factor bromocriptine which disrupt prolactin secretion, and cloprostenol a PGF2α analogue which acts directly with the CL. FSH levels were comparable to in all treatment groups. However there was a reduction in LH after treatment with cloprostenol compared to LH levels observed in the control and bromocriptine animals, although due to the large variations observed in LH levels this reduction was not significant (Figure 6.10).

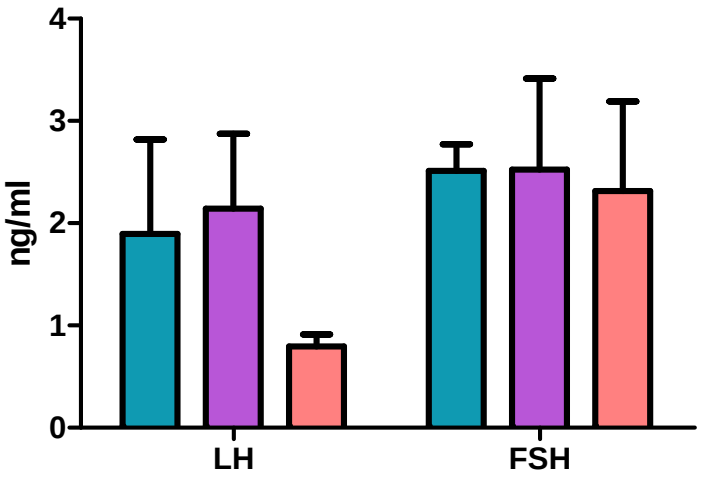

Figure 6.10: Changes in mean (±SEM) plasma concentrations of LH and FSH in non-treated pregnant controls and after luteolytic induction treatments bromocriptine and cloprostenol.

6.4. Discussion

6.4.1. PDCD4 as a putative DAZL target

This study is the first to identify PDCD4 not only as a potential DAZL mRNA target but additionally reports the temporal expression of PDCD4 within ovarian tissue. The initial identification of PDCD4 led to an extensive investigation in an attempt to elucidate probable PDCD4 functions within the ovary, with particular reference to the CL. The identification of the DAZL consensus sequence within the mouse PDCD4 homologue suggests a potential role for this gene in DAZL-mediated regulation and control. As previously investigated (Chapter 3 and 4), the functional copy number of DAZL is believed to be affecting the productivity of folliculogenesis and subsequently increasing the rate of ovulation and hence number of offspring in the Hets. Comparison of PDCD4 mRNA between Wt and Het mice, no differences were detectable between PDCD4 mRNA levels in d21 oocytes or d10 ovaries were observed, suggesting the control mechanisms of the mRNA expression are not altered as a result of reduced functional copy of DAZL. Although, it cannot be completely ruled out that PDCD4 is a functional target of DAZL until *in vivo* interactions are disproven. This will require further optimisation of the immunoprecipitation technique (Chapter 4) for ovarian tissue in addition to investigating different approaches altogether to determine *in vivo* interactions.

6.4.2. Potential ovarian function of PDCD4

There are limited publications describing the expression of PDCD4 within the ovary. It has been detected within the oocyte and throughout blasotcyst development in the mouse, and the study states that its function is unknown although associated with apoptosis (Jurisicova et al., 1998). Furthermore, from a microarray study comparing human ovarian tumours, PDCD4 mRNA expression was shown to be notably decreased. Again elucidation of its function was not discussed (Bonome et al., 2005). However, this decrease in expression may be due to cellular transformation and progression of tumour growth rather than representative of a role for PDCD4 in ovarian function. Therefore we report here for the first time the temporal expression of PDCD4 within the mouse ovary, in addition to suggesting potential involvement of this protein in functional luteolysis. Evidence has been presented that PDCD4 is

detected at the mRNA and protein level in oocytes, which is supported by previous findings (Jurisicova et al., 1998), and additionally, protein has been localised to granulosa cells, the ovarian surface epithelium, oviduct epithelium and luteinised granulosa cells. Due to this extensive expression of PDCD4 in the female reproductive tissue in addition to its host of roles in pro-apoptotic mechanisms (Shibahara et al., 1995), the likelihood of PDCD4 being a direct target of the oocyte specific DAZL remains low. However PDCD4 may well play a significant role in the control and development of both the oocytes and surrounding ovarian structures. This chapter has highlighted that there are still many mechanisms within the ovary which remain unsolved and with regards to intra/extracellular control of CL during luteolysis the striking nuclear expression of PDCD4 cannot be overlooked. To understand the possible ovarian function of PDCD4 an understanding in to its functional role elsewhere has been examined and discussed.

6.4.3. Function of PDCD4

PDCD4 was originally isolated in a search for genes that were activated as a result of apoptosis (Shibahara et al., 1995). In addition work investigating species homology indicates that PDCD4 is highly conserved during evolution indicating its importance in biological function (Lankat-Buttgereit et al., 2004) and has been related to a variety of functions within different tissue and cell systems but how it actually works is still to be fully elucidated. More recently, interests lies in the observation that PDCD4 has a tumor suppressor functioning by targeting and inhibiting translation and transformation. It has been identified that translation initiation factor (eIF4A) is a major binding component of PDCD4 (Yang et al., 2003) and like the translation initiation factor 4G (eIF4G), PDCD4 can interact with the RNA helicase eIF4A via its component MA3 domains. This competitive binding between PDCD4 and eIF4G results in an inhibition of translation by interfering with the assembly of the initiation complex consisting of the translation factors eIF4G, eIF4E and eIF4A (Goke et al., 2002; Yang et al., 2003). As a result, it seems that in the absence of PDCD4, eIF4A would be left unchecked to increase the amount of inefficiently translated mRNAs, thereby causing the cells to proliferate aberrantly leading to cancer (Jansen et al., 2005). It is important to note that this initiation complex has also been implicated in

the control of DAZL mediate transcription in Xenopus, whereby DAZL binds to Poly (A) binding proteins which are actively involved in the translation initiation complex (Gray et al., 2000; Collier et al., 2005).

Studies performed in PDCD4 deficient mice support the role of PDCD4 as a suppressor of tumorgenesis *in vivo* and additionally indicate its involvement in inflammation initiation by selectively inhibiting protein translation in the immune system (Hilliard et al., 2006). It has been shown in culture that the expression of PDCD4 is dramatically up-regulated in association with early event apoptosis as a result of serum starvation. Furthermore, it has been suggested that PDCD4 may be involved in both the intrinsic and extrinsic pathways of apoptosis (Goke et al., 2002).

Interestingly, unlike other documented translation factors that are constitutively expressed under physiological conditions, PDCD4 is normally expressed at low levels but is dramatically up-regulated under conditions that induce programmed cell death or transformation (Shibahara et al., 1995; Onishi et al., 1998; Goke et al., 2002). Therefore, the differences observed in PDCD4 immunoexpression at the time of functional luteolysis within the CL sequentially determine how much functional translation suppression is occurring regarding protein initiation and hence molecular function of PDCD4.

In the present study PDCD4 was predominately localised to the cytoplasm of granulosa cells of both healthy and atretic follicles and oocytes, with the notable difference in cellular compartmentalisation observed in the CL, most probably as a result of stage of regression and luteinisation. Thus, the differences observed further suggest that PDCD4 is an active molecular component within the CL, almost certainly associated with cell death. In light of these findings and in support of the difference observed, PDCD4 has been reported to be localised to the cytoplasm (Yang et al., 2003) and to the nucleus (Schlichter et al., 2001a; b). In a subsequent study PDCD4 protein was detected in the cytoplasm and/or in the nucleus, depending on the cells or their growth state (Yoshinaga et al., 1999). Collectively these

observations suggest that in addition to the reported roles of PDCD4 in protein translation within the cytoplasm, PDCD4 may have an important functional role within the nucleus. To clarify PDCD4 localisation, a further (Bohm et al., 2003) investigation revealed that PDCD4 can actually shuttle between the cytoplasm and nucleus, with nuclear location dominantly selected under normal growth conditions. In contrast to normal growth conditions, in serum depleted cells PDCD4 protein is located in the cytoplasm (Bohm et al., 2003). These findings suggest that this transfer in localisation from the cytoplasm to the nucleus is responsible for active translation inhibition thereby initiating apoptosis. In contradiction to this, it has been suggested that nuclear accumulation of PDCD4 occurs prior to apoptosis (Zhang et al., 2006). More recently it has been identified that phosphorylation of PCDC4 by Akt is the cause of the nuclear translocation (Palamarchuk et al., 2005) and phosphorylation of PDCD4 *in vitro* and *in vivo* occurs in a P13K-dependent manner, causing nuclear translocation. This action of phosphorlation inactivates PDCD4 in its function as an inhibitor of AP-1 mediated transcription. Members of the TGFβ superfamily have also been implicated in PDCD4 regulation, with PDCD4 increasing after treatment of transfected PDCD4 Huh7 cells with transforming growth factor-β. This increase in PDCD4 not only coincided with the occurrence of apoptosis and caspase activation but was reversed by the transfection of Smad7 (a known antagonist of TGFβ1) suggesting that Smad7 protect cells from TGF β1 mediated apoptosis (Zhang et al., 2006). Although somewhat under investigated, these studies suggest that PDCD4 may function in both the nucleus and the cytoplasm, with nuclear expression associated with normal growth and cytoplasmic expression associated with apoptosis. Furthermore, translocation from the cytoplasm to the nucleus may be associated with inactivation of PDCD4 and initiation of eIF4G transcription.

A coincidental common linkage has been formed between catenins and cadherins which have been identified both as putative DAZL targets as part of the bioinformatics trawl in Chapter 5, and to PDCD4. It has been shown that knockdown of PDCD4 expression stimulates the translocation of β-catenin into the nuclei, which in turn activates β-cateninsol T cell factor (Tcf)-dependent

transcription, and AP-1 dependent transcription. Thus, it has been suggested that PDCD4 regulates the expression of E-cadherin since both mRNA and protein levels of E-cadherin are decreased as a result of PDCD4 knock down. Additionally E-cadherin is a binding partner of β-catenin and therefore a decrease in E-cadherin expression resulted in an increase of cytoplasmic free β-catenin (Wang et al., 2007). Given that these two proteins, β-catenin and E-cadherin, are both potentially regulated by PDCD4 and catenin-1 and N-cadherin have been identified as putative targets of DAZL, communication between these groups of molecules may be linked and, although not by a directly functional interaction, may play important roles in translational control of interacting mechanisms.

More recently novel findings have identified that suppression of PDCD4 expression is vital for the invasive activity of COX-2 mediated by PGE (2) and IL-8, and that PDCD4 increases TIMP-2 expression to inhibit breast cancer cell invasion (Nieves-Alicea et al., 2008). COX-2, TIMPs and PGE are all recognised as regulatory factors within the CL and most certainly play a dominant role in luteolysis (Bardin, 1970; Smith et al., 1999; Wiltbank and Ottobre, 2003). It is well established that prostaglandins are involved in initiation of luteolysis in a number of species including rodents (Bardin, 1970; Smith et al., 1999; Wiltbank and Ottobre, 2003). With these data a plausible function for PDCD4 within the CL may be that increased expression associated with cytoplasmic localisation could increase TIMP 2 and hence aid structural regression of the CL. This suggestion is supported by studies performed in rats where the expression of TIMP-2 mRNA in the regressing CL suggests an involvement in luteal demise (Simpson et al., 2001).

The identification and molecular function of PDCD4 are recent discoveries and despite the documentation of potential roles the molecular function remains to be fully investigated, although some aspects of its function are beginning to emerge. The main functions remain to be associated with protein translation with PDCD4 and eIF4G having similar homolog thus being involved in translation suppression within the cytoplasmic compartment. This localisation is enhanced as a direct result of serum starvation, inducing apoptosis, with nuclear representation associated with

normal growth. All these documented mechanisms are transferable to the ovary, and in particular to the CL, where we observe an increase in cytoplasmic expression of PDCD4 as a result of removal of the luteolytic effects of prolactin. This increase in PDCD4 cytoplasmic intensity is accompanied by an increase in nuclear expression and further augments the possibilities of multiple functions within different cellular compartments. In addition to these findings it has also been shown that PDCD4 has intrinsic RNA binding properties although this has not been extensively investigated (Bohm et al., 2003). We can suggest that cytoplasmic expression is aiding the pro-apoptotic mechanisms of PDCD4 whereby protein translation is hindered allowing programmed cell death to occur. Furthermore nuclear expression is exacerbating the signalling outcomes and could be contributing indirectly to the cell fate. Even though this is the first study suggesting an active role of PDCD4 within the CL, the identity of RNA targets would additionally aid the clarification of the mechanistic properties of PDCD4. In conclusion these speculative suggestions require functional confirmation, with the need to amalgamate all the potential associated systems to collate an overall theory of PDCD4 function and apply them to the ovarian requirements.

6.4.4. PCNA v BrdU

PCNA and BrdU were utilised in the current studies as markers of cell proliferation, and as comparative factors for PDCD4 immunoexpression. In all studies, a large proportion of cells were actively labelled for PCNA compared to the BrdU. It has been recognized that PCNA can be continuously expressed in cells that are not actively dividing possibly due to its long half-life (Morris and Mathews 1989; Scott et al., 1991). PCNA has also been shown to be involved in DNA repair, which is not directly associated with proliferation (Celis and Madsen, 1986; Toschi and Bravo, 1988; Shivji et al., 1992; Wood and Shivji, 1997), which could also have contributed to the fact that a high proportion of cells were PCNA labelled compared to BrdU.

6.4.5. Apoptosis of the ovary

In the ovary the critical decisions between cell survival and cell death involve extensive communication between groups of pro-apoptotic and pro-survival

molecules. It is believed that this highly regulated coordination is entirely a quality control mechanism, supporting the viable oocyte with potential to ovulate and to eliminate those oocytes and follicles that become atretic. It is well documented that programmed cell death plays an important role in the regulation of ovarian maintenance with controlled cell death present at numerous stages of oogenesis and folliculogenesis. DAZL is undoubtedly essential for oocyte survival and by mechanisms not yet identified it may be contributing to the fate of the oocytes since, in the absence of DAZL, oocytes are lost after birth in mice. DAZL therefore could possibly be linked to factors initiating cell survival, including possible interactions with PDCD4. Thus we investigated the potential involvement of PDCD4 in ovarian apoptotic mechanisms, in both follicle atresia and luteolysis of the CL.

Alternative treatment regimes were designed to initiate follicle atresia by FSH withdrawal and to initiate functional luteolysis by the administration of two known luteolytic agents, bromocriptine and cloprostenol. FSH withdrawal undoubtedly induced follicle atresia in ovaries of d21 mice with extensive up-regulation of cleaved caspase 3 protein expression. As a result of this onset of apoptosis, cleaved caspase 3 mRNA was reduced following FSH withdrawal suggesting functional transcription had ceased and cell death was occurring. Even though PDCD4 has been targeted as a pro-apoptotic factor, induced upon apoptotic initiation, there was no difference in mRNA or protein expression in response to FSH withdrawal. One suggestion is that although PDCD4 was present in the cytoplasm in the developing follicle it remains functionally quiescent at the onset of rapid withdrawal of FSH as alternative death mediated pathways are recruited. Comparatively, follicle atresia is a quick and efficient process occurring rapidly within the ovary continuously whereas luteolysis is the gradual regression of the CL, which is both a functional and morphological process.

From the visual analysis of pro-estrous ovaries and after luteolytic treatment, it can be concluded that CLs are present at different stages of progression and regression with luteolysis occurring at different rates. PDCD4 phosphorylation (Palamarchuk et al., 2005) and hence translocation of functional protein to the nucleus may be

involved in the slow programmed cell death observed in the CL via shuttling between cytoplasm and nucleus. PDCD4 protein expression was remarkably different between CLs in the same ovary possibly due to the stage of regression of each CL. Not only did the intensity of cytoplasmic expression alter, nuclear expression was observed. Taking in to account that both old CLs from previous cycles and new CLs from the current cycle are present in the pro-estrous ovaries and not supporting pregnancy with their progesterone secretion, nuclear localisation of PDCD4 was inversely correlated with the expression of cleaved caspase 3. It became apparent that when PDCD4 was nuclear in the examined CL, cleaved caspase 3 expression was barely detectable. However when there was an increase in cytoplasmic expression and reduced nuclear expression of PDCD4, suggesting an increase in translation inhibition, there was a marked expression of cleaved caspase 3 cells within these regressing CLs. These observations suggest that caspase 3 is functionally required for apoptosis to proceed normally during luteal regression but that enhancement of PDCD4 within the cytoplasm may aid the onset of cleaved caspase 3 expression with both contributing to luteolytic degradation (Carambula et al., 2002). Extending this investigation into the inducement of functional and structural regression of the CL, by inducement of luteolysis, a similar pattern of PDCD4 and cleaved caspase 3 was observed. Again it was notable that there was increased intensity of PDCD4 both nuclear and cytoplasmic staining upon inducement of luteolysis whether by bromocriptine or cloprostenol emphasising a potential functional role in programmed cell death within the CL. As previously mentioned cleaved caspase 3 was only observed in CL in which there was a decrease in nuclear PDCD4 expression, and only in the CL from the bromocriptine and pro-oestrous models.

Finally, despite the original identification of PDCD4 mRNA as a potential target for DAZL mediated functions it has become evident that this novel transcription suppressor may be playing a significant role in luteolysis. The abundant expression and localisation of PDCD4 in the CL may be key finding in regulating and facilitating events within the CL of the mature ovary. One can now propose that there is a possible role for PDCD4 in programmed cell death associated with

structural CL regression. The observed difference in PDCD4 localisation from high cytoplasmic expression and nuclear localisation may be associated with the caspase cascade induction. In contrast, in follicles it has been shown that PDCD4 is probably not involved in cell death as no difference in protein or mRNA levels were detected as a result of natural or induced follicle atresia. The mechanisms and control of PDCD4 now require further investigation to explore the possible mechanisms of this protein and to determine whether PDCD4 plays a substantial role in preventing and controlling cell proliferation by means of mediated control over programmed cell death.

Chapter 7: *In vitro* maturation of Wt and Het follicles

7.1 Introduction

Investigating the molecular mechanisms by which DAZL functions directly has been explored previously in Chapters 3 and 4, looking at identifying oocyte affected genes and potential mRNA targets. Attempting to identify target mRNAs has led to a plethora of potential candidates including those identified in Chapter 5 (Venables et al., 2001; Jiao et al., 2002; Maegawa et al., 2002; Fox et al., 2005; Reynolds et al., 2005: Reynolds et al., 2007) and with the mRNA binding properties of the DAZL protein (Cooke et al., 1996) it may, as previously suggested, have multiple targets with multiple functional effects. It is now recognised (Cooke et al., 1996) that murine DAZL gene function is essential for germ cell development. In addition there is now preliminary evidence (Chapter 3) that functional copy number does have an effect on additional oocyte gene regulation. Surprisingly, the presence of only one functional copy of DAZL is somehow exerting an effect on the fecundity of the heterozygous females (McNeilly et al., 2000). The present chapter now focuses on the indirect action that DAZL may be having on the ovarian follicle unit as a whole, investigating the bi-directional communication between the oocyte and somatic cells of the follicle which in turn may lead to identifying biochemical pathways involved in the increased follicle development and ovulation in the DAZL Het females.

In addition to Het female mice producing significantly larger numbers of viable offspring compared to Wt, it has also been previously shown that Het females have increased plasma Inhibin B and reduced FSH levels (McNeilly et al., 2000). Furthermore, follicle growth has been reported to be significantly increased in the Het animals in culture when compared to the growth of the Wt follicles (McNeilly et al., SSR 2007). This increase in growth occurred in *in vitro* cultures supplemented with FSH at concentrations of 1iu, 0.1iu and 0.01iu, indicative of an increase in sensitivity of the Het follicles to FSH in *in vitro* culture follicles (McNeilly et al., SSR 2007). Initial stereological follicle count analysis indicated that at d21 the Het ovary contains a significantly larger proportion of large antral follicles compared to the Wt. This proportional increase observed in the Hets can be reversed after

administration of ovine follicular fluid (source of inhibin) to that of the Wt numbers. One proposed suggestion is that in addition to the Het ovary containing a greater number of advanced follicles compared to that of the Wt of the same age, atresia rates are reduced. The combination of these two events in the Het females, advanced follicle stimulation with reduced atresia rates, would explain the increased litter sizes. However, previous findings do not support the second part of this theory (Watson, PhD 2007), since atresia rates assessed by the presence of the apoptotic marker cleaved-caspase 3 and stereological analysis were not significantly different between the Het and Wt aged day 21.

Since it is now recognised that bi-directional communication is vital to oocyte maturation and follicle development, it seems apparent that DAZL may be taking part in controlling various pathways involved in follicle selection, development and survival. Follicle survival is an extremely inefficient process whereby the majority of ovarian follicles are actually lost either prior to birth or for those which do succeed into postnatal life, are mainly lost prior to ovulation, with only a few highly selected successful follicles making it to ovulation. It remains unclear what is controlling this selection process but intense competition between female gametes or at least a rigorous selection process must be in place (Hartshorne, 1997). This regulation of follicle survival may be one of the factors contributing to the difference observed between the DAZL phenotypes, whereby there is a reduction in follicle competition and reduced selection criteria in the Hets which may be controlled by local regulatory factors.

The events associated with follicle growth are highly regulated and controlled by many local and systemic endocrine, molecular and cellular organisational factors. Many of these key mechanisms interact and are synchronised to achieve the ultimate goal of follicle maturation and consequently successful ovulation (Chapter 1). Growth of and hormone secretion by follicles from DAZL mice was assessed in culture in previous studies in our laboratory (McNeilly et al., SSR 2007; Watson, PhD 2007) but molecular factors have not been investigated.

The main aim of the studies in this chapter were to describe the molecular differences between Wt and Het follicles in culture by investigating the indirect actions DAZL gene expression on follicle growth, maintenance and differentiation from the FSH independent or responsive, to the FSH dependent stage. Understanding the endocrine control, follicle sensitivity to gonadotrophins and successive growth rates in addition to investigating mRNAs expressed in the follicle to assess maturation and responsiveness are fundamental to understand these underlying mechanisms. To accomplish this, *in vitro* culture of individually isolated follicles in varying FSH treatments was performed closely mimicking *in vivo* follicle development. The growth rates of the follicles were recorded over a six day period after which follicles were collected individually for comparative gene expression analysis. To address the gonadotrophin independent to responsive stages of follicle growth, collections of classified (un-cultured) follicles were analysed for the same follicle expressing genes.

7.2 Materials and Methods

7.2.1 Follicle culture methods

In vivo follicle culture systems are an essential valuable tool in the process of understanding the underlying mechanisms of the oocyte/follicle growth and differentiation. As a guide it is estimated that only up to 12 follicles may be dissected and be appropriate for culture per mouse, six per ovary aged 21-25 days, but this number varied considerably depending on the individual mouse. Individual follicles were dissected from Wt and Het DAZL mice and further selected for culture. Isolation of individual follicles allows accurate observation of the growth and changes in gross morphology of follicles. Although mechanical isolation of individual follicles is a laborious task it does ensure that individual follicles are at a similar stage of development.

Follicle culture techniques were not established previously at the Queens Medical Research Institute. Therefore initial training was undertaken with Dr Alison Murray within Dr Norah Spears' Laboratory, George Square, University of Edinburgh (Spears et al., 1998). The equipment and technique were subsequently set up and managed successfully by myself at the MRC HRSU, Edinburgh.

7.2.2 Follicle Dissection

All dissections were carried out under aseptic conditions in a laminar flow hood (Jencons). With the microscope positioned far enough in the hood to protect the tissue and provided uninterrupted airflow. For the *in vitro* primary follicles cultures, both animal and bench were sprayed with 70% ethanol and instruments used cleaned thoroughly in virkon (Antec International) and 70% ethanol prior to use to minimise the risk of contamination to the culture system. The ovaries are removed from the mouse and placed into warmed L-15 medium (adjusted to 285 mOsm/kg using sterile water (Sigma) using an osmometer (Roebling) supplemented with 0.3% w/v BSA (Sigma Aldrich, Poole UK). Under a microscope the ovaries are cleared of any surrounding fat and adhering tissue using insulin needles (Sharwood-Davies, UK cat no-1100601630). Each ovary halved and each piece placed into a fresh dish of L-15,

keep in the incubator at 37°C, only half an ovary was dissected at a time for a maximum of 15 minutes.

7.2.3 Follicle isolation

Under a dissecting microscope (Zeiss KL1500 LCD) with a heated stage (Linkam Scientific Instruments) set at 37°C the Wt and Het ovaries were mechanically dissected using 27-gauge syringe needles and the appropriate sized follicles approx 180-200µm in diameter were selected. Any adhering stroma was removed from follicles. After 15 mins of dissection follicles were transferred to a fresh dish of L-15 medium. The harvested preantral follicles were then evaluated, and those exhibiting an intact basement membrane, a high density of granulosa cell layers, centrally placed oocytes with some thecal attachment were selected for further culture. Any follicles presenting with irregularities such as being non-spherical, having a non-central oocyte or having any darkening of granulosa cells as a result of atresia were avoided.

7.2.4 Follicle culture

Isolated Wt and Het follicles were cultured individually in a round bottom 96 well plate (non TC treated, Iwaki plates-cat no 3875-096; lids cat no 1803-096) equilibrated in an incubator (37°C 5% CO2) for 1-16h. Each follicle was placed in 30µl of culture medium in the bottom of the first row of wells and overlaid with 75µl silicone fluid (Dow-Corning cat no 630064v).

Culture Media (1ml):

935 ml Alpha-Mem (Gibco-22571)

10 µl FSH (1 iu rhFSH; Purgon 50iu/0.5ml)

50µl serum (5%)

5µl ascorbic acid (Sigma) (made as 5mg/ml sodium ascorbate in alpha-mem)

The medium was sterilised using small iwaki filter (13mm cat no 2032-014 for volumes under 10ml).

The follicles were cultured in four treatment groups (0.01iu; 0.1iu; 0.5iu and 1iu FSH (Puregon, Organon)) for a six day period after which follicles were individually collected for gene profiling analysis. The follicles were moved to fresh medium daily and the diameter was measuring using an eye piece graticule and recorded. If the follicle was not round the average was taken from perpendicular width and length measurements. All manipulations of the follicles are carried out using finely drawn Pasture pipettes coated with 0.1% BSA (Sigma). Follicles that did not survive the six day culture period, those with obvious darkening of the granulosa cell mass and/or signs of disintegration or that had spontaneously extruded their oocytes, were not included in the analysis. These observations of follicle loss were likely due to manual damage caused during the isolation process.

7.2.5 RNA extraction

RNA was extracted from individual follicles using the Qiagen microRNAeasy protocol (Section 2.3). The standard protocol was adapted to some extent as the volume of starting material was so small, and to increase the probability of the follicle being homogenised. Hence, follicle samples were homogenised in a reduced volume of 100µl of RLT (normal volume 350µl) before being made up to the required 350µl. The extracted RNA was then reverse transcribed to produce cDNA, which was subsequently used for expression analysis (Section 2.3.4).

7.2.6 Gene expression

Taqman gene expression assays were used to quantify mRNA expression levels of selected genes in individual follicles (Table 2.4). Initial optimisation shows that when using limited material like individual follicle where typical mRNA yields were 10ng/µl the gene expression was surprisingly still within the threshold limits of gene expression, 18s (<23) and Ct (<36) value (Section 2.4.2).

7.2.7 Statistics

Results for growth and atresia rates from were from at least five independent experimental culture runs, with the size of follicles in each group averaged, (1iu FSH Wt n=9, Het n=54; 0.5iu FSH Wt n=38, Het n=49; 0.1iu Wt n=20, Het n=49 and

0.01iu FSH Wt n=50; Het n=52). Data from follicle growth were analysed using student's t-test comparing Wt and Het on each day of growth. $\chi 2$ was used to determine the atretic significance between Wt and Het follicles. For taqman gene expression 10 representative follicle from each sample group were selected and each sample was repeated in triplicate and compared to 18s expression. The results were analysed using one way ANOVA with Bonferroni's multiple comparison test and student's t-test comparing Wt and Het of the different treatment groups.

7.3 Results

7.3.1 Follicle culture

Follicles from treatment groups 0.1iu, 0.5iu and 1iu FSH had similar morphology at day six of culture (Figure 7.1). These large follicles were spherical in shape, surrounded by an intact layer of theca cells with clear areas identifiable in the dense granulosa cells layers indicative of antral formation with the oocyte marginally visible within the central region.

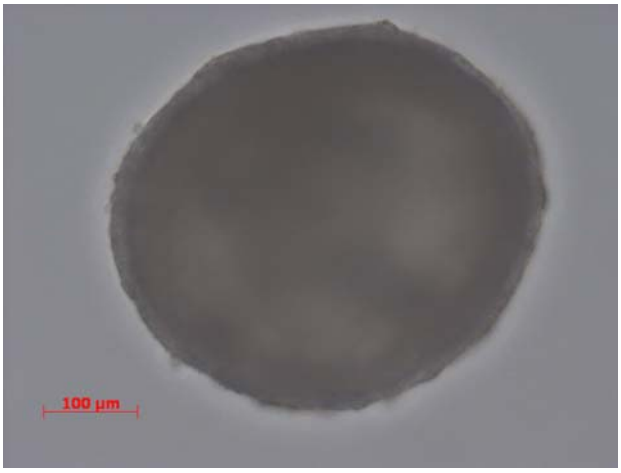

Figure 7.1: Manually isolated mouse antral follicle approximately 400 µm in diameter growing *in vitro* in culture (1iu FSH after six days). Scale bar represents 100 µm.

7.3.2 Follicle growth

Follicle growth is not only an indication of gonadotrophin responsiveness but cellular differentiation and overall follicle maturation. When observing the four treatment groups there was no difference in final diameter between Wt and Het follicles at 1iu, 0.5iu or 0.1iu (Figures 7.2). At 0.5iu FSH Het follicle were significantly larger ($P<0.01$ day 1-2, $P<0.05$ day 3 and $P<0.01$ day 4) in size between day one and for, but there was no difference is final size at day six. At 0.1iu FSH, Wt follicles were significantly larger ($P<0.05$ and $P<0.01$ consecutively) at day three and day five the Het (Figure 7.2). However with 0.01iu FSH the Het follicles were significantly larger in diameter by day three ($P<0.01$, Figure 7.2) and remained significantly ($P<0.01$) larger over the remainder of the culture period. Results from this study have provided evidence and support that follicle growth does significantly differ with

the presence of one functional copy DAZL compared to two copies in the Wt response to low doses (0.01iu) of FSH.

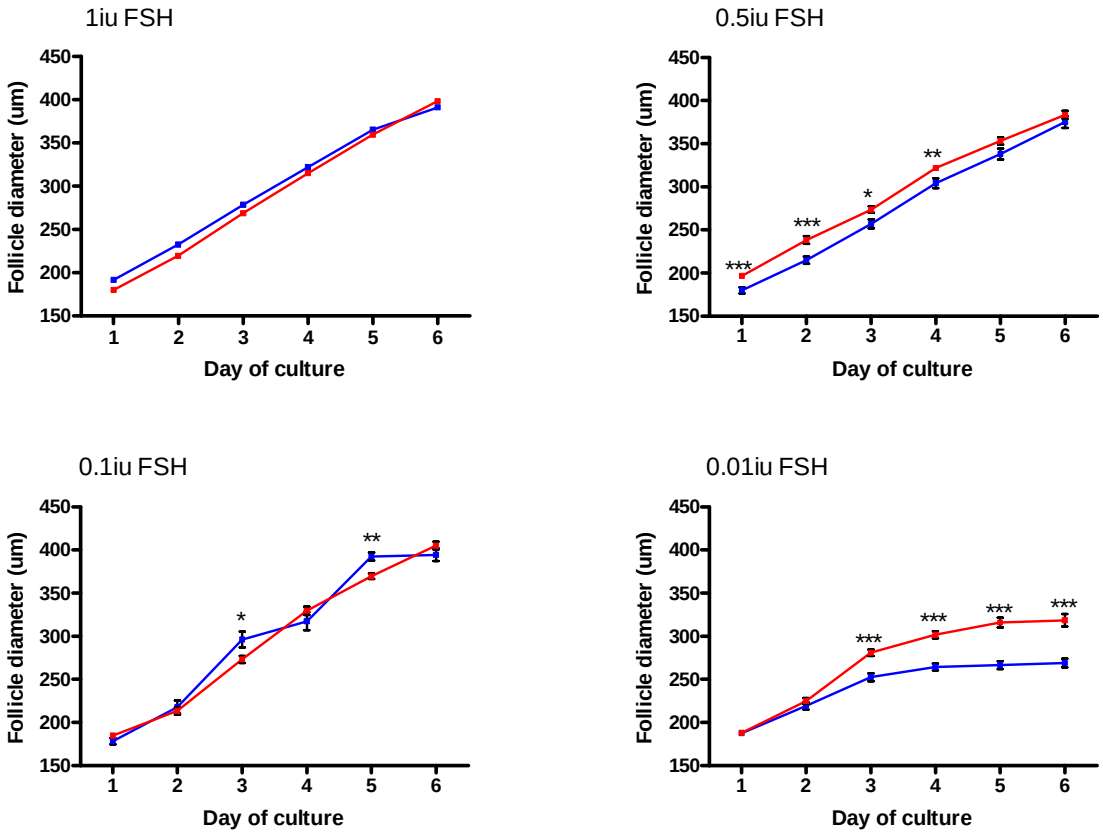

Figure 7.2: Follicle growth over a six day culture of Wt v Het at 1iu, 0.5u, 0.1iu and 0.01iu FSH. (Values are means ± SEM (n ≥ 9) *P<0.05, **P<0.01 and ***P<0.01). Wt and Het .

When comparing the Wt and Het over the range of FSH concentrations, final follicle sizes are comparable at 1iu, 0.5iu and 0.1iu for both Wt and Het (Figure 7.3). It may be that the endogenous FSH supplied at these doses are surplus to follicle demand and the cultured follicles are exhibiting saturation as differences in growth rate are not significantly different. The perturbed growth effect observed at 0.01iu suggests that FSH is playing an important role in maintenance of follicle growth and when in limited supply follicle growth is compromised, but more so in the Wt follicles.

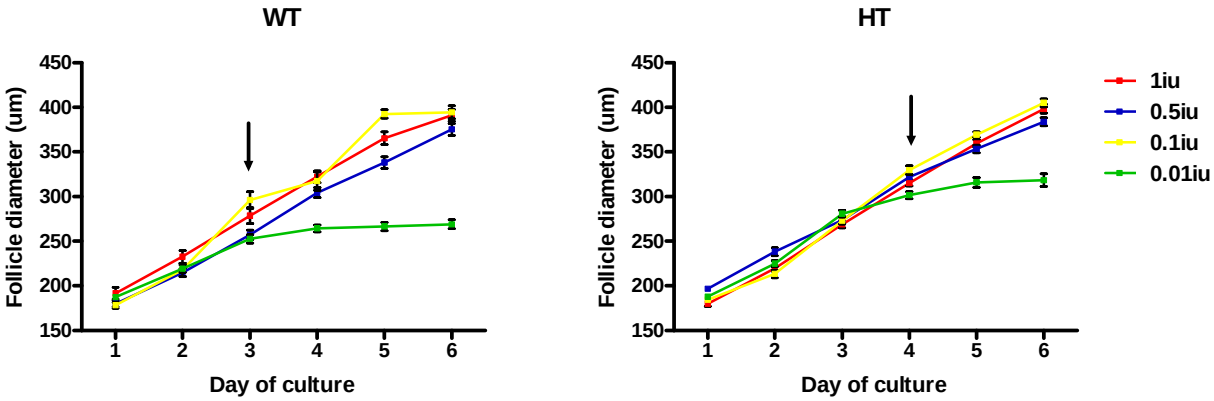

Figure 7.3: Follicle growth of Wt and Het follicles cultured in the presence of 1iu, 0.5iu, 0.1iu and 0.01iu recombinant FSH. Black arrows indicates cessation of follicles growth in 0.01iu FSH culture conditions (day 3 Wt and day 4 Het). Values are means ±SEM.

When directly comparing growth rates within a genotype at the lowest (0.01iu) FSH dose it can be observed that follicle growth ceases after three days in culture for Wt and four days for the Hets (Figure 7.3). In addition this prolonged growth of the Het at 0.01iu FSH was accompanied by antral formation in a small proportion of Het follicles. However this maturation stage was never observed within the Wt follicle cultured in 0.01iu conditions, suggesting there is insufficient FSH present to meet the demands for antral formation.

7.3.3 Rate of follicle atresia

In addition to monitoring daily follicle growth, the rates of follicle atresia were also observed (Figure 7.4). The classification of follicle atresia in culture was defined as a shrinking and overall darkening of the granulosa cells surrounding the oocyte, and these follicles ceased to undergo further development. There was a notable reduction in the total percentage of Het follicles undergoing atresia compared to Wt follicle at all concentrations of added FSH. At 1iu FSH 20% of Wt follicle were classified as atretic compared to 4% of Het ($P<0.05$), at 0.5iu FSH 7% of Wt compared to 1% of Het (result not significant), at 0.1iu FSH 23% of Wt compared to 11% of Het ($P<0.05$) and finally 18% Wt compared to 2% of Het ($P<0.01$).

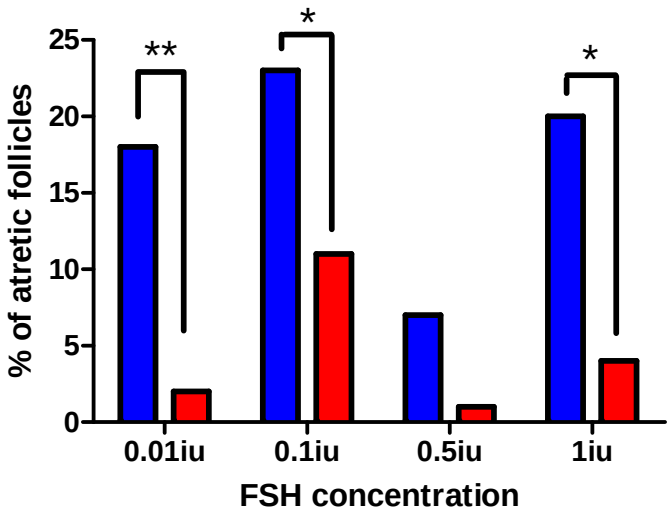

Figure 7.4: The percentage of atretic follicles at the end of six days in culture for Wt and Het follicles in 0.01iu, 0.1iu, 0.5iu and 1iu FSH. (Values are overall % of total follicles cultured and monitored *p<0.05 and **p<0.01). Wt and Het .

7.3.4 Gene analysis of cultured follicles

mRNA expression levels of selected gene were analysed and compared from individual follicles from culture 1iu, 0.5iu and 0.1iu FSH. Growth by day six was perturbed in follicles from the 0.01iu treatment these were eliminated from the gene expression analysis.

7.3.4.1.Gonadotrophin receptors and aromatase

FSH receptor (FSHR) mRNA levels were investigated between the Wt and Het follicles, there being a significantly ($P<0.05$) up-regulation in the Het mice compared to the Wt at 1iu FSH (Figure 7.5). This up-regulation was not observed at 0.1iu and 0.5iu FSH culture conditions, although there was a reduction in FSHR mRNA in the Het compared to the Wt at 0.5iu FSH this result was not significant due to the high variation between samples.

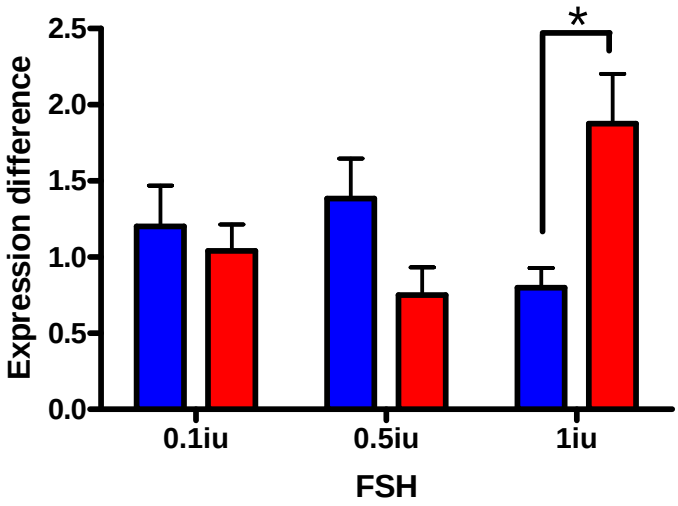

Figure 7.5: Mean fold differences of FSHR for Wt and Het DAZL follicles cultures for six consecutive days in 1iu, 0.5iu and 0.1iu concentrations of FSH. Values are the means ± SEM relative to calibrator sample * $p<0.05$. Wt and Het .

LHR mRNA expression levels were analysed and detected in all the three treatment groups (Figure 7.6). The genotype of the DAZL mice did not have a significant effect of mRNA expression. There was a reduction in LHR mRNA expression in both Wt and Het follicles at the 0.1iu FSH compared to the levels detected at 1iu and 0.5iu, but this result was not significant.

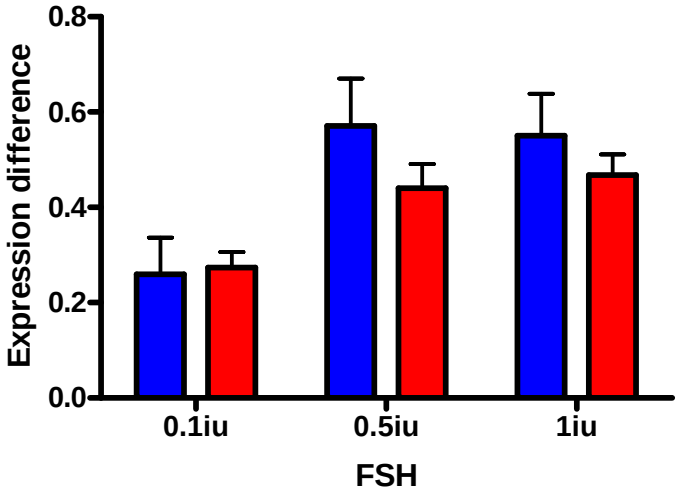

Figure 7.6: Mean expression differences of LHR for Wt and Het DAZL follicles cultures for six consecutive days in 0.1iu, 0.5iu and 1iu concentrations of FSH. (Values are the means ± SEM relative to calibrator sample). Wt and Het .

Aromatase mRNA expression was comparable at 0.1iu and 1iu FSH culture conditions between the Wt and Het follicles (Figure 7.7). However there was a significant ($P<0.05$) up-regulation in expression of aromatase mRNA at 0.5iu FSH in the Het follicles compared to the Wt follicles.

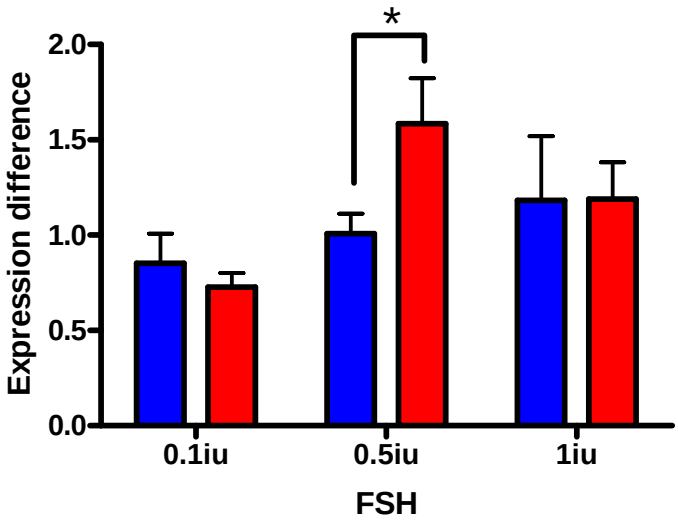

Figure 7.7: Mean fold differences of Aromatase for Wt and Het DAZL follicles cultures for 6 consecutive days in 1iu, 0.5iu and 0.1iu concentrations of FSH. Values are the means ± SEM relative to calibrator sample * $p<0.05$. Wt and Het .

7.3.4.2.TGF β superfamily

Members of the TGFβ superfamily are widely expressed within the ovary and developing follicle and influence growth and function in a developmentally regulated manner. These proteins have important roles in the control of folliculogenesis despite the majority of functional studies being mostly *in vivo*. In the present study, Anti-Mullarian Hormone (AMH), Follistatin (FST), and inhibin and activin subunit (βA, βB and α) mRNA levels were all selected for investigated between the culture groups.

AMH is considered to have an essential role in folliculogenesis inhibiting the process of follicle recruitment and modifies the growth of preantral and antral follicles by diminishing the sensitivity of follicles for FSH stimulation. It may be expected because of this that difference might occur between the Het and Wt. However AMH mRNA expression showed no difference between genotypes at the different culture conditions (Figure 7.8). There was a tendency for increased mRNA expression at 0.1iu and 1iu in the Het follicles but due to sample variation these results were not significant.

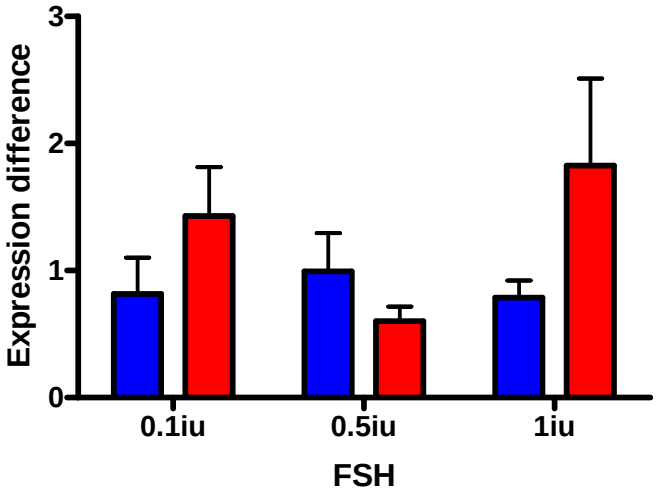

Figure 7.8: Mean fold differences of AMH for Wt and Het DAZL follicles cultures for 6 consecutive days in 1iu, 0.5iu and 0.1iu concentrations of FSH. Values are the means ± SEM relative to calibrator sample. Wt and Het .

FST mRNA expression was similar between genotypes at each FSH concentration (Figure 7.9). mRNA expression was reduced when the culture FSH concentration was reduced to 0.01iu FSH. Using comparative one way ANOVA this reduction in both Wt and Het is significantly different compared to mRNA expression at 1iu and 0.5iu. There is a significant increase ($P<0.05$) in FST mRNA expression in correlation with an increase in concentration of FSH in culture of both Wt and Het at 0.5iu and 1iu FSH compared to 0.1iu.

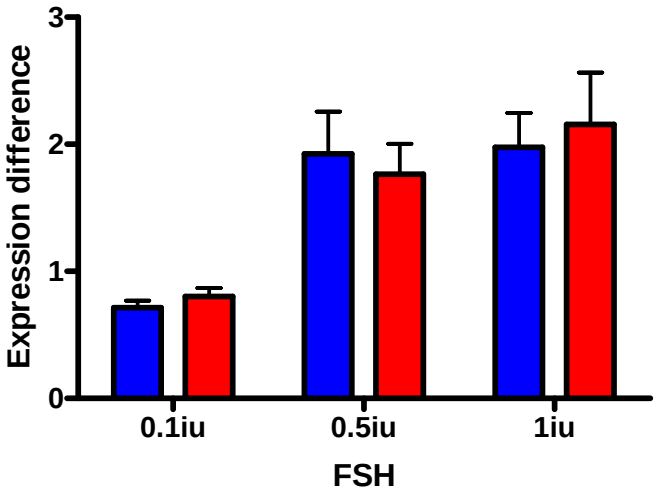

Figure 7.9: Mean fold differences of FST for Wt and Het DAZL follicles cultures for 6 consecutive days in 1iu, 0.5iu and 0.1iu concentrations of FSH. Values are the means ± SEM relative to calibrator sample. Wt and Het .

Expression of AR11a mRNA was just detectable for quantification but, with the results at the maximum limit for detection, the accuracy of the results may be jeopardised. Although not significant there was a trend for a dose dependent increase in AR11a mRNA expression as a result of increased FSH concentrations in the Het follicle cultures, with no change in the Wt.

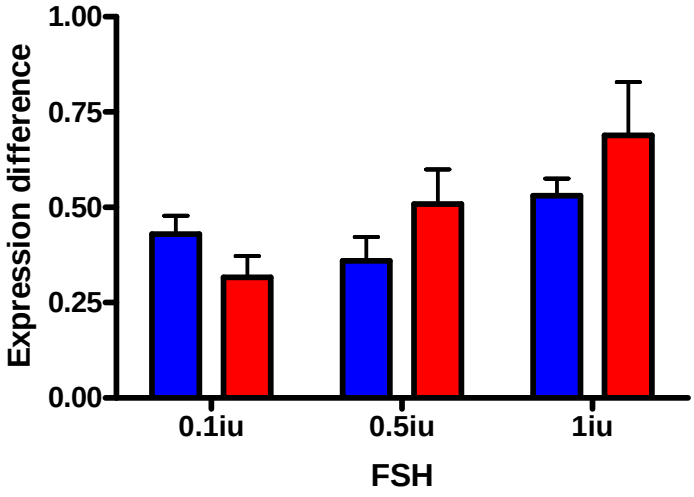

Figure 7.10: Mean fold differences of AR11a for Wt and Het DAZL follicles cultures for 6 consecutive days in 1iu, 0.5iu and 0.1iu concentrations of FSH. Values are the means ± SEM relative to calibrator sample. Wt ■ and Het ■.

The changes in mRNA for inhibin α, inhibin βB, inhibin βA subunits were analysed. The expression of inhibin α mRNA did not differ between culture groups and was not different between genotypes (Figure 7.11).

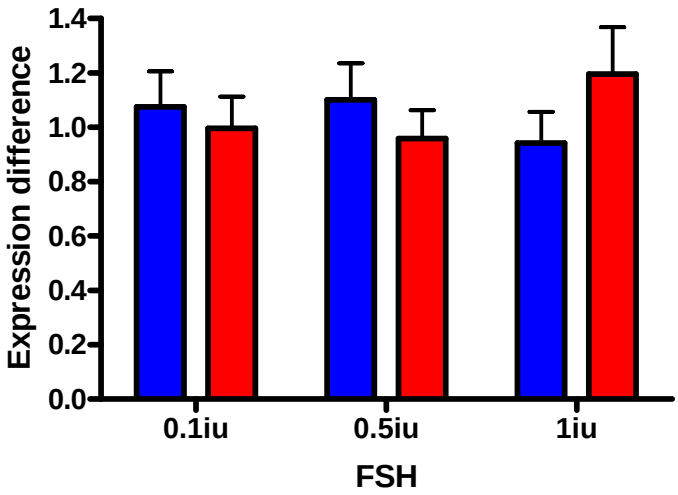

Figure 7.11: Mean fold differences of Inhibinα for Wt and Het DAZL follicles cultures for six consecutive days in 1iu, 0.5iu and 0.1iu concentrations of FSH. Values are the means ± SEM relative to calibrator sample. Wt and Het .

The level of mRNA of inhibin βB subunit was significantly reduced (P<0.01) in the Het follicles at 0.5iu FSH compared to that of the Wt (Figure 7.12). For Wt follicles inhibin βB mRNA levels were significantly higher (P<0.05) at 0.5iu compared with both 1iu and 0.01iu FSH. In contrast there were no differences between FSH treatments in Het follicles.

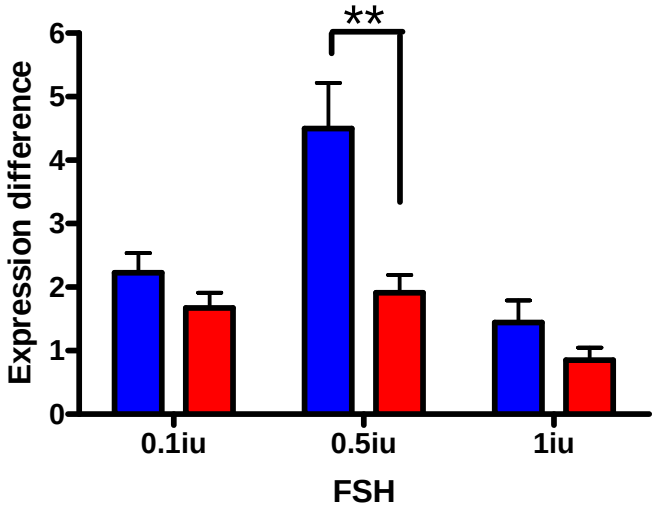

Figure 7.12: Mean fold differences of inhibin βB for Wt and Het DAZL follicles cultures for six consecutive days in 1iu, 0.5iu and 0.1iu concentrations of FSH. Values are the means ± SEM relative to calibrator sample ** p<0.01. Wt and Het .

In contrast to βB subunit mRNA the inhibin βA subunit mRNA were significantly ($P<0.05$) increased in the Het follicles compared to the Wt at 0.5iu FSH (Figure 7.13). Furthermore, for Het follicles inhibin βA mRNA levels were significantly higher ($P<0.05$) at 0.5iu compared with both 1iu and 0.01iu of FSH. In contrast there were no differences between inhibin βA mRNA levels and FSH treatments in Wt follicles.

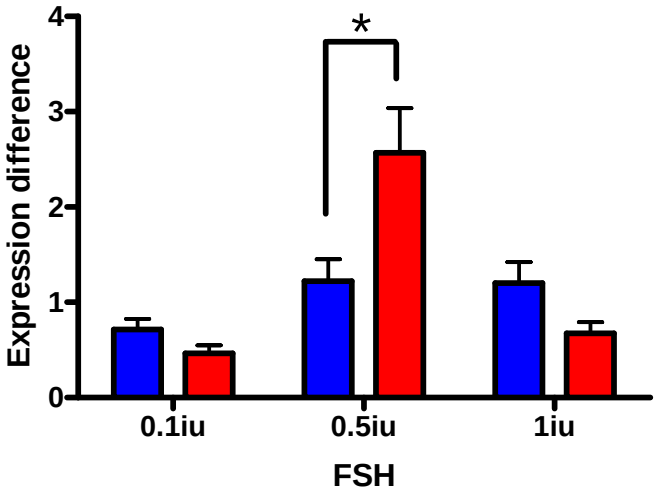

Figure 7.13: Mean fold differences of Inhibin βA for Wt and Het DAZL follicles cultures for six consecutive days in 1iu, 0.5iu and 0.1iu concentrations of FSH. Values are the means ± SEM relative to calibrator sample * $p<0.05$. Wt and Het .

7.3.5 Gene analysis of gonadotrophin independent stage

7.3.5.1.Gonadotrophin receptors

Real-time taqman PCR analysis was used to measure gene expression in follicles at the gonadotrophin independent stage of growth (≤180μm), prior to antral development. These follicles were collected directly from the ovaries and were not cultured. Cohorts of five follicles were classified into size groups from 60μm-180μm (n=3 for each group).

There were no differences between FSHR and LHR mRNA expression between genotypes as the follicle size increased (Figure 7.14; 7.15).

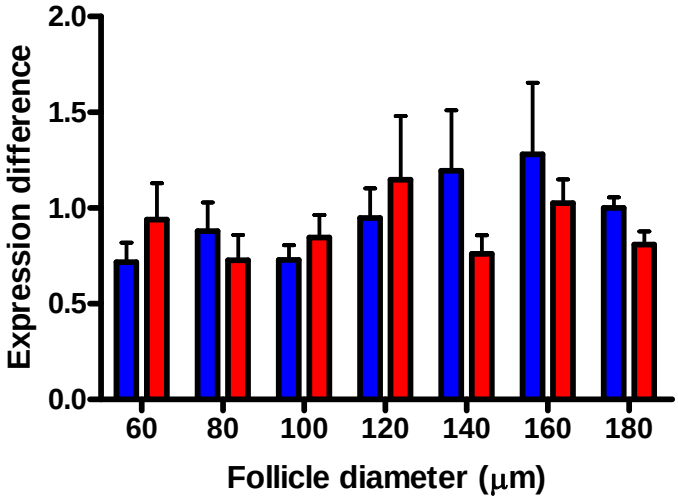

Figure 7.14: Mean fold differences of FSHR for DAZL follicles at gonadotrophin independent stage from sizes 60μm to 180 μm. Values are the means ± SEM relative to calibrator sample. Wt and Het .

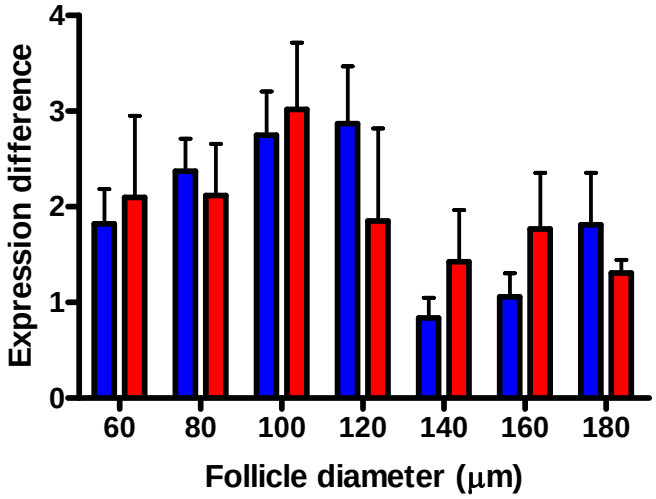

Figure 7.15: Mean fold differences of LHR for DAZL follicles at gonadotrophin independent stage from sizes 60μm to 180 μm. Values are the means ± SEM relative to calibrator sample. Wt and Het .

7.3.5.2.Inhibin subunits

Analysis of mRNA levels for the inhibin subunits, inhibin α, inhibin βB and inhibin βA which form the basis of activin and inhibin molecules from sized follicles did not show any significant differences between genotypes at each follicle stage. Inhibin βA mRNA expression appears decreased as follicles became more gonadotrophin responsive but these changes were not significant (Figures 7.16; 7.17; 7.18).

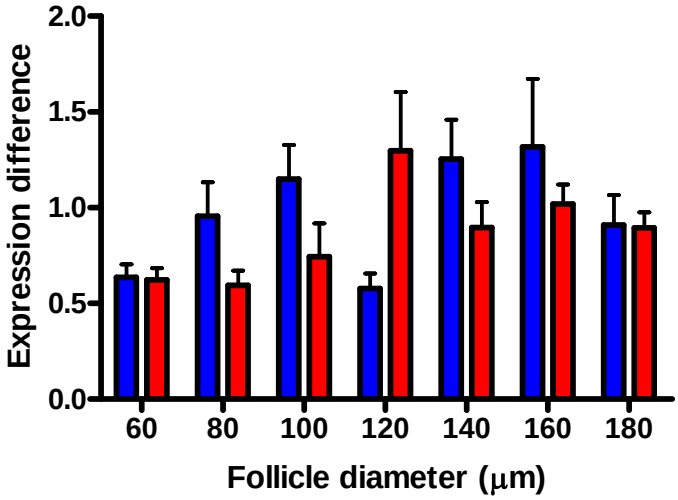

Figure 7.16: Mean fold differences of inhibin α for DAZL follicles at gonadotrophin independent stage from sizes 60μm to 180 μm. Values are the means ± SEM relative to calibrator sample. Wt and Het .

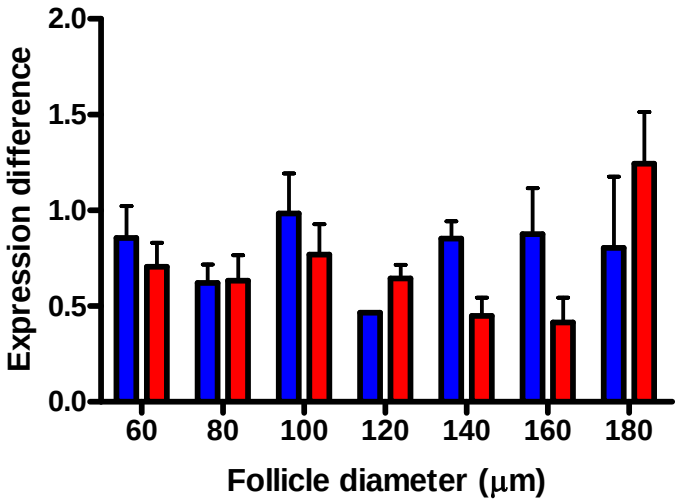

Figure 7.17: Mean fold differences of inhibin βB for DAZL follicles at gonadotrophin independent stage from sizes 60μm to 180 μm. Values are the means ± SEM relative to calibrator sample. Wt and Het .

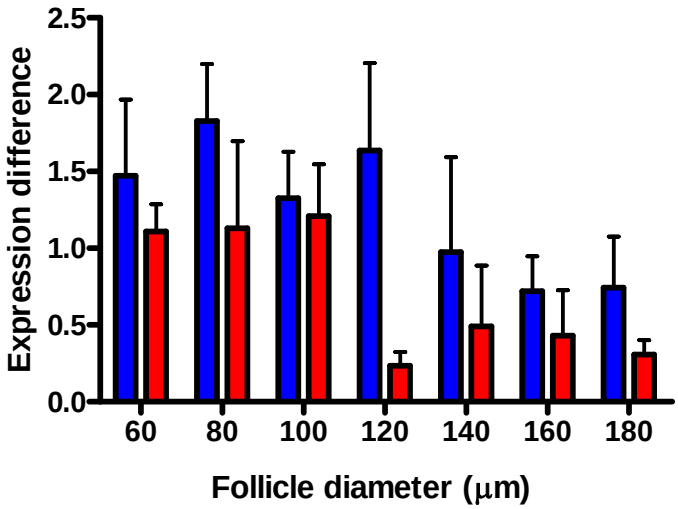

Figure 7.18: Mean fold differences of inhibin βA for DAZL follicles at gonadotrophin independent stage from sizes 60μm to 180 μm. Values are the means ± SEM relative to calibrator sample. Wt and Het .

Table 7.1: Summary of follicle gene expression differences between Wt and Het

***In vitro* (<340 µm)**	**0.1iu FSH**	**0.5iu FSH**	**1iu FSH**
FSH receptor	ND	ND	Het>Wt
LH receptor	ND	ND	ND
Aromatase	ND	Wt<Het	ND
AMH	ND	ND	ND
FST	0.1iu<0.5iu & 1iu	ND	ND
AR11a	ND	ND	ND
Inhibin βA	ND	Wt<Het	ND
Inhibin βB	ND	Wt>Het	ND
Inhibin α	ND	ND	ND

In vivo (>180)	**60µm**	**80 µm**	**100 µm**	**120 µm**	**140 µm**	**160 µm**	**180 µm**
FSH receptor	There was no significant difference between the Wt and Het genotypes at any stage of development in the gonadotrophin independent stage to the gonadotrophin responsive stage for the genes investigated.						
LH receptor							
Inhibin βA							
Inhibin βB							
Inhibin α							

* ND= no significant difference

7.4 Discussion

The primary aim of this chapter was to investigate the indirect actions of functional DAZL gene expression. To accomplish this, follicle growth was monitored first and, although it had been previously reported to be different between the Wt and Het follicles, verification of the results was required. Secondly atresia rates of follicles in culture were monitored as these had not been previously investigated. Finally, follicle gene expression was investigated to show any response differences as a result of the genotype and FSH stimulation. Understanding the endocrine control, follicle sensitivity to gonadotrophins and successive growth rates in addition to investigating mRNAs expressed in follicles to assess maturation and responsiveness are fundamental in understanding the underlying mechanisms whereby DAZL heterozygous expression increases fertility.

7.4.1 Follicle growth

This study demonstrated and confirmed that *in vitro* Het follicles are more sensitive to low concentrations of FSH compared to Wt follicles. Follicles selected for culture had established their growth phase having progressed *in vivo* from primordial to the early pre-antral stages, already appearing with thecal or stromal cells attached to the exterior of the follicle wall. Follicles under the influence of 1iu, 0.5iu and 0.1iu FSH in culture all reached the same maximum size of approximately 430 μm after six days in culture, only slightly smaller than pre-ovulatory follicle found *in vivo*. This maximum size of follicles is comparable to ranges (>500μm) previously reported for mouse follicles in culture (Hartshorne et al., 1994; Spears et al., 1998; Murray et al., 2008). It has been established that FSH is required for full follicle development in culture (Spears et al., 1998).

At a high dose (1iu FSH) follicles from both genotypes grew substantially over the six day period at the same rate. Reducing the FSH concentration to 0.5iu Het follicles grew at a significantly faster rate for the initial four days compared to the Wt, but by day five there was no differences and by the collection stage at day six follicles from both genotypes were equivalent in morphology and size. These findings suggest that at a reduced concentration of FSH (as observed from 1iu to

0.5iu) Het follicles have accelerated growth in the initial stages. It would therefore be reasonable to assume at 0.1iu this observed difference might be exaggerated and thus a larger difference between the growth rate of the Het and Wt follicles may occur. However, although reaching comparable final size in actual fact Wt follicles were significantly larger than Het follicles on day three and day five of culture. These findings do not support the FSH sensitivity and threshold theory as previously observed, but the current studies used a different preparation of recombinant human FSH than the earlier studies in our laboratory, hence the concentration of FSH in culture was reduced further. Under the influence of this reduced FSH (0.01iu) Wt follicle only grew for the initial three days to a size of approximately 250μm before ceasing to develop further. In contrast, Het follicle grew for four days reaching a size of approximately 275μm reaching a maximum of 300μm by day six, thus clearly indicating that Het follicles are more sensitive to FSH than Wt at 0.01iu FSH. Although not completely comparable to previous findings (Elaine Watson, PhD 2007), whereby significant difference in growth between the Het and Wt were observed at 1iu, these data still support a difference in follicle sensitivity. Furthermore on explanation for the differences observed are that the present study used FSH in a liquid preparation whereas the previous study (Elaine Watson, PhD 2007) used a suspension, possibly altering the bioavailability of FSH to the growing follicle. In addition, fetal calf serum was used in the present study compared to HPG mouse serum, although both have been shown to provide the correct supplementation for growth (Boland et al., 1993; Cortvrindt et al., 1996). These results further confirm that FSH is essential for follicle growth and maturation *in vitro* and by reducing FSH the growth of follicle is compromised even without considering the genotype. Although differences were observed in growth rates in the current studies these would need to be confirmed *in vivo* to have physiological relevance.

FSH in the *in vitro* culture system stimulates glucose utilisation and oestradiol production and may also stimulate follicles to enlarge or granulosa cell density to increase (Nayudu and Osborn 1992; Boland et al, 1994; Hartshorne et al., 1994). It is well established that *in vivo* FSH is essential for early antral formation and follicle growth and differentiation. The cessation of follicle growth in addition to the lack of

antral formation in the Wt follicles at 0.01iu FSH confirms that FSH is indeed crucial for these processes. Further supporting the importance of FSH are two transgenic models, the FSHβ deficient mouse and the FSHR KO which are both infertile, confirming that the function of FSH is vital for fertility and ovarian function (Kumar et al., 1997; Abel et al., 2000). In comparison, it has also been recognised that excessive exposure to FSH could result in the down-regulation of the FSHR, leading to suboptimal follicular response (LaPolt et al., 1992), again supporting the theory that the FSH concentration is critical for normal development. It is worth noting that follicles cultured in high dose 1iu FSH may be down-regulating the FSHR expression hence the increased in atresia rate compared to 0.5iu FSH.

Despite follicle culture being an invaluable tool to aid biological research, it is not without its flaws. There is evidence that the growth rates of the granulosa cell layers in *in vitro* cultures exceed the ability of the surrounding thecal layers to maintain adequate contacts between the thecal layer, basement membrane and granulosa cells, resulting in the occasional bursting or bulging of the follicle (Hartshorne, 1997). Bursting/bulging of follicles was observed in the present study, in relation to inadequate cellular growth but, in addition, mechanical manipulation may also contribute to these events. Another common observation within *in vitro* follicle culture system is of slow and abnormal growth of follicles which results in the overgrowth of the surrounding theca (Nayudu and Osborn, 1992). Again poor selection of follicles when preparing the cultures or failure to remove excess stromal tissue, may also add to this.

It is important to note that ovarian follicles in static culture conditions exist in a very different environment in comparison to the dymanic situation *in vivo*. It has been previously acknowledged that the growth rate of cultured follicles is accelerated *in vitro* compared to naturally occurring growth *in vivo* (Pedersen, 1970). This expedited growth may be due to several factors, including the removal of the follicle from its ovarian niche where inhibitory influences produced by other growing follicles are absent, or as a direct result of the artificial culture conditions taking into account osmotic pressure changes and absence of intraovarian pressure (DiZerega et

al., 1983: Qvist et al., 1990: Nayudu and Osborn 1992). Furthermore, the maximum size that follicles can achieve *in vitro* have physical constraints compared to that *in vivo*, the major factor being the absence of a rich blood supply to the theca. This rich blood supply transports the systemic gonadotrophins, particularly just before ovulation (Zeleznik et al., 1981) and is involved in supplying localised steroid substrates, growth factors and structural support to the granulosa compartment (Skinner and Coffey, 1988). In comparison the granulosa cell layer is avascular with the oocytes acquiring only limited oxygen via diffusion suggesting the interior of the follicle structure is quite hypoxic, even *in vivo* (Boland et al., 1994). This hypoxic state may contribute to the ability of the follicular oocytes to survive *in vitro* as they are already adapted and tolerant to the gaseous conditions (Hartshorne, 1997).

7.4.2 Follilce atresia

Follicle atresia is a natural phenomenon occurring continuously within the ovary and it is not surprising that when removing follicles from their internal ovarian niche atresia rates differ. One major contributing factor to follicle atresia is the competition for follicle dominance and response to FSH. It has been established that there is a critical widow of sensitive whereby the follicle responds appropriately depending on its fate (McGee and Hsueh, 2000). In addition to cultured follicles having accelerated growth (Pedersen, 1970), the proportion of follicles avoiding atresia *in vitro* appears to be greater than that *in vivo* although not studied extensively it may be due to factors including physiological concentrations of gonadotrophins and reduced competition due to individual isolation.

For the first time this study has shown that atresia rates are significantly different between the Wt and Het cultured follicles. The susceptibility of the Wt follicles to become atretic in culture suggests that the follicles are less tolerant to the culture conditions and are possibly less responsive to the protective roles of FSH. The Wt follicles may also be more prone to atresia due to the altered expression of regulatory factors, including growth factors or intra-ovarian signals which may be below threshold levels. The molecular control of this increased atresia rate by DAZL in Wt follicles may be contributing to the observed differences in litter sizes. Furthermore,

from these studies it an be concluded the genotype but possibly not FSH concentration are having an effect on overall atresia rate further supporting the hypothesis that FSH works within a critical window of bioavailability to balance follicle selection, growth and atresia which may be altered between the Wt and Het.

These experiments investigating the responses of individually cultured follicles from Wt and Het females to their FSH environment has shown that follicles are developmentally competent to undergo apoptosis-mediated cell death from the preantral/ early antral stage onward, at least *in vitro*, substantiating previous reports (McGee et al., 1997). Furthermore, experiments using rat follicles cultured in the absence of hormones showed a 12-fold increase in the level of apoptotic DNA fragmentation (Chun et al., 1996). This apoptotic event was prevented by treatment with FSH in a dose-dependent manner, these results supporting FSH as a major survival factor for early antral follicles. However, in the current studies the decrease in FSH levels did not subsequently reduce atresia rates further as one may expect. Additionally, if all follicles are classified as dominant as a consequence of no intra-ovarian competition, it is believed that the dominant follicle survives the declining plasma FSH due to their enhanced responsiveness to gonadotrophins relative to that of subordinate follicles (Ginther et al., 1996; 2001). Therefore, the observed atresia rates in *in vitro* matured follicles are a true genotype phenomenon and not a result of declining culture FSH. The Het follicles not only survive the decline in FSH from 1iu to 0.01iu, they have increased responsiveness to FSH, and hence enhanced growth at low levels of FSH. The reduction in follicular growth response at low dose FSH supported the previous findings that there is altered sensitivity within the Het compared to the Wt follicles. Finally these results suggest that the cohort of follicles recruited at each oestrous cycle within the Het ovary have a higher proportion of dominance and greater capacity to avoid atresia compared to the Wt.

As previously discussed (Chapter 3) there is evidence that oocyte genes may be targeted and affected as a result of active DAZL gene expression. Additionally the results from the current study show that the growth rates of Wt and Het follicles are similar at high dose FSH (1iu-0.1iu) treatment groups. Thus, an investigation into

local follicle gene expression was performed. Furthermore regardless of DAZL being germ cell specific, the results from the other studies reported in this thesis suggest that DAZL gene functionality is having an effect on the whole follicle.

7.4.3 Gene analysis of cultured follicles

Follicle growth can be categorised into stage specific phases, the gonadotrophin independent, gonadotrophin responsive and the gonadotrophin dependent stages. At each of these stages a range of factors regulate the growth and differentiation of the oocyte, granulosa and the thecal layers. Many follicles will leave the gonadotrophin independent stage and become responsive, conversely many will not mature fully but instead will undergo atresia during this developmental process. The criteria for follicles to achieve the position of dominance and become gonadotrophin dependent are mainly controlled by a tightly regulated feedback system. The current study was used to investigate the expression of components of this important feedback regulatory system to determine, if any, constraints or enhancements are present between the Wt and Het follicles.

7.4.3.1.Gonadotrophin receptors and aromatase

As previously noted FSH and LH are fundamental for providing the correct conditions for follicle stimulation and ovulation. Therefore analysis of the gonadotrophin receptor expression FSHR and LHR was investigated to determine whether the threshold sensitivity to gonadotrophins was a direct result of possible altered receptor expression and enhanced follicle maturity.

The foremost function of FSH is to stimulate granulosa cell proliferation and is involved in the positive feedback expression and function of its own receptor. It has been controversial whether or not FSH does actually regulate its own receptor, but early reports do state that up and down-regulation of the receptor can occur after FSH stimulation (Richards, 1980). Members of the TGFβ family have also been implicated in controlling the expression of the FSHR gene and the formation of the receptors. FSHR are exclusively expressed by granulosa cells from as early as the two-layer or primary stage of follicle development (Oktay et al., 1997) with oocytes

influenced and affected indirectly. During antral formation FSH acts upon its receptor in the granulosa cell surface membrane to stimulate cell division and formation of glycosaminoglycans that are essential components of antral fluid (Hillier, 1991). Therefore, the results from the mRNA analysis in the current study indicate that if FSH is up-regulating its own receptor at specific stages, evidence from the Het follicles at 1iu FSH and not the Wt, further supports the hypothesis that Het follicles are more sensitive to FSH. Furthermore, the increase of FSHR at 1iu in the Het may be a result of a greater number of granulosa cells expressing the FSHR at that stage in growth, or the number of granulosa cells is greater for the same give follicle size. In addition these data suggests that DAZL may be playing a role in bi-directional communication between its oocyte cytoplasmic location and subsequent function on FSHR on the granulosa cells. Although detected, LHR mRNA expression was unchanged from the Wt and Het follicles suggesting that the follicles may be at the same stage of responsiveness as LHR increases on theca and on the outer layer of granulosa cells in preovulatory follicles prior to the LH surge.

It is well established that aromatase is involved in oestrogen biosynthesis which plays an obligatory role in follicle dominance and endocrine control. Our data shows that there is a significant increase in aromatase mRNA expression in the Het follicles compared to the Wt follicles at 0.5iu FSH supporting the role of aromatase in follicle dominance. Furthermore, previous studies have shown that oestradiol production of cultured follicles at 1iu of FSH to not significantly differ between the Wt and Het suggesting equal dominance despite accelerated growth in the Het (Elaine Watson, PhD 2007). However, the oestradiol production was not analysed from cultures at lower FSH stimulations, where differences in aromatase mRNA expression were observed in the present study. This now requires further investigation to establish if the Het follicles are more dominant compared to Wt and lower doses of FSH.

7.4.3.2.TGFβ superfamily

Folliculogenesis requires the coordinated regulation of many genes, and members of the TGFβ family have been implicated in critical events associated with determining the fate of follicle survival. This family of proteins are classified as multifunctional cytokines and control events such as cellular proliferation, differentiation and interactions with the extra cellular matrix. The inhibins, activins and follistatins were first identified over 30 years ago in ovarian follicular fluid (deJong and Sharpe, 1976) due to their ability to modulate FSH secretion. It is now widely acknowledged that activin and inhibin play significant roles in follicle development (Knight and Glister, 2006). It therefore seemed logical that activin and inhibin signalling molecules were assessed to establish whether there was any difference in responsiveness or particular feedback systems. In *in vitro* situations inhibins and follistatin have been show to suppress FSH secretion, whereas the activins augment FSH secretion (Knight, 1996; Mather et al., 1997; de Kretser et al., 2000; Knight and Glister, 2001). Comparatively and in response to the FSH secretion pattern it has been demonstrated in cattle that over the stage of follicle development the actions of activins decrease, whereas the actions of inhibins increase as does the actions of follistatin (Knight and Glister, 2001).

FST is structurally unrelated to the inhibins despite having similar function in suppressing pituitary FSH (Ueno et al., 1987). In addition FST binds to the activins and too lesser extent inhibins through the β subunit, naturalising their functional activity (Shimasaki et al., 1991), with the binding affinity of FST for activin similar to that of activin binding to its own receptors (Mathews, 1994). FST mRNA was not found to change in the follicles, with very little variation between treatment groups and genotype. There are reports using bovine follicles which show that intrafollicular concentrations of FST do not vary throughout growth, hence suggesting a developmental regulated bio-availability of activin A, due to the effects free activin A/inhibin (Knight and Glister, 2003) and a similar mechanism may be in place in the mouse.

As mentioned the functions of inhibin and activin change as follicle progression occurs. Therefore the investigation in to their subunit expression was performed to attempt to elucidate if the function of these critical endocrine components were altered in Het compared to Wt follicles. It has been previously shown that as follicles increase in size inhibin A increases whilst the inhibin B decreases (Knight and Glister, 2003). Thus, the ratio of inhibin A: B rises and peaks at ovulation accompanied by no change in FST and an increase in activin A (Schwall et al., 1990; Yamoto et al., 1992; Roberts et al., 1993; Knight and Glister, 2003). The inhibin α subunit is expressed throughout all follicle stages with expression increasing in the large antral follicles. Furthermore, the inhibin βA subunit is expressed in the theca cells of the secondary to large antral follicles (Gougeon 1994; Hsueh et al., 1996; Bristol and Woodruff, 2004) and by the granulosa cells of primordial and primary follicles as is the inhibin βB subunit. The roles of the inhibins and activins undoubtedly are crucial for correct follicle development (Knight and Glister, 2003; 2006) and, therefore, it is not surprising that there was significant difference observed between the cultured Wt and Het follicles. Findings from this study show that inhibin βB mRNA expression was reduced in the Het follicles compared to the Wt, suggesting an overall decrease in activin AB/B or inhibin B production which in turn can be interpreted as the Het follicles being more advanced, although comparable in size to the Wt. Moreover, inhibin βA mRNA expression was increased in the Het follicle compared to the Wt directing inhibin/activin production towards activin A or inhibin A, which again supports the concept that Het follicles are at a more advanced stage of development. There was no change between genotypes for the inhibin α subunit.

It has been suggested that AMH has two main functions within the ovary firstly inhibiting initial follicle recruitment and secondly acting as an inhibitory growth factor during early stages of folliculogenesis by preventing the stimulatory effect of FSH on primordial follicle growth (Durlinger et al., 2002). Due to the large variation in results it difficult to predict if AMH mRNA expression is having an effect as an indirect result of the genotype. It could be proposed that if indeed it is acting as a primordial follicle inhibitor, it may be expected that there be a reduced function in

the Het follicles allowing for increased recruitment. To address this issue, earlier follicles would be required for analysis.

7.4.4 Gene analysis of gonadotrophin independent stage

The growing follicle undergoes considerable morphological changes which are associated with stage-specific roles of genes throughout folliculogenesis. Events which occur are extensive and include: alterations in size due to proliferation, recruitment and differentiation of somatic cells, the accumulation of fluid within the antral cavity and alterations in oocyte size, associated with the maturing nucleus and cytoplamic components (Bownes and Gosden, 2005).

Having identified potential factors including FSHR, LHR and the inhibin subunits, which may be involved in the phenotypic Het effect in the initial part of this study, it was important to analyse follicles from earlier stages of development to determine the stage at which control mechanisms may become altered. The factors analysed are developmentally regulated and identifying when and where changes may occur is critical to the advancement of understanding of why DAZL functional copy number is having such a conspicuous effect on rate of preovulatory follicle development and ovulation *in vivo*.

The assessment of the aforementioned mRNA expression in the cohorts of classified follicles remains inconclusive, most notably due to the large variations observed between samples. In general it could be suggested that the effects of DAZL gene expression are only visible at the gonadotrophin dependent stage of development, yet it is dubious to make such a bold statement. It is important to note that with all experiments the same concentration of RNA was used but the composition of RNA may be different due to the structure of the follicle with larger follicles containing more granulosa cells, thecal cells and a larger oocyte compare to smaller follicles, thus contributing to the large variations observed. Further analysis is now required to assess the selected factors at these early gonadotrophin independent to gonadotrophin responsive stages.

7.4.5 Conclusion

DAZL is undoubtedly essential for germ cell development with the functional consequences of the KO model being very distinctive with loss of all oocytes at birth (Ruggiu et al., 1996). It is not uncommon for KO models of germ cell or, indeed, of ovary genes to be infertile, but it is scientifically intriguing why the heterozygous DAZL KO mice should have such a positive phenotype of increased ovulation and hence litter size. We know that the oocytes of the DAZL KO are present prior to birth and at meiosis, malfunction of the germ cells takes place and oocyte ablation occurs (Ruggiu et al., 1996). It could be suggested, although there is no evidence that this fetal effect would also affect the Hets at this same stage, thus, resulting in a programming event making the Het follicles more responsive for selected ovulation and hence have increased sensitivity to FSH. Additionally, results from this study show that the mechanisms of atresia associated with follicle survival are also altered in the Hets compared to Wt.

FSH concentration understandably affects growth potential with threshold limits being the desired mechanism for selected receptor expression regulation and subsequent gonadotrophin function. There is a vast amount of evidence to suggest that inhibins, activins and follistatin function as intra-ovarian regulatory molecules involved in many processes including somatic cell proliferation, steroidogenesis, oocyte maturation and CL formation (Knight and Glister, 2006). If these regulatory molecules are altered as an indirect result of DAZL function within the ovary it may explain or partially explain the Het phenotype. These regulatory pathways at each stage of development now require a more comprehensive study to fully explain the difference observed between the phenotypes. If particular systems have an increase in sensitivity, or communication is enhanced, this may be having the observable effects detected in this study relating to increase fertility. Growth factors that augment FSH responsiveness would promote further follicle development, whereas ones that attenuate FSH responsiveness might induce atresia, this may be the situation that is occurring in the DAZL Het, whereby a growth factor is enhancing follicle growth and reducing atresia.

Furthermore, no histological analysis was performed on cultured follicles, therefore further investigating the differences in oocyte size/quality, prevalence of apoptosis, granulosa cell number and ability for cumulus expansion and overall maturation of the oocyte may highlight differenced in the morphological development of the Hets follicles in addition to the molecular control.

Endocrine and locally produced factors are essential requirements for successful follicular growth and maturation, the process which is highly regulated and demanding. It can now be added that components of this controlled developmental process are altered in the Hets allowing increased follicle advancement and affecting sensitivity to FSH and inadvertently may be subsequently affecting the ease of fertilisation. These altered systems may result from fetal life development during meiosis of the primordial germ cells or it may be that DAZL is functionally active within the adult ovary through a mechanism yet to be discovered. The RNA binding properties of DAZL are inconclusive with many potential targets (Venables et al. 2001; Jiao et al. 2002; Maegawa et al. 2002; Fox et al. 2005; Reynolds et al. 2005; Reynolds et al., 2007) including those identified in Chapter 5, none of which would so far explain the hypothesis partially proven in this thesis, that ovarian sensitivity is altered. An understanding of how the increased responsiveness of heterozygous follicle is accomplished, and how the differences in signalling are achieved under the coordinated response in terms of ordered follicular development to ovulatory status, or to selectively reduce follicle atresia remains a major goal. Understanding the underlying phenomenon that increase litter sizes remains elusive although progress has now been made in directing the control to the gonadotrophin dependent stage of development involving bi-directional communication between the oocyte and granulosa cells and implicating FSHR, aromatase and members of the of TGFβ superfamily.

Chapter 8: Final discussion

The studies in this thesis stem from an interest in ovarian failure and the mechanisms by which it can arise. A large proportion of human female reproductive disorders are caused by unknown factors with only a minority being identified to be caused by genetic mutations. Therefore, genes which cause ovarian failure in mice are potential candidates for ovarian failure in humans, and there are now many transgenic murine ovarian pathologies which closely resembling conditions observed in humans. Consequently, the aims of this thesis were to establish the direct and indirect actions of the oocyte specific gene DAZL on the molecular mechanisms associated with ovarian regulation and control. Moreover, interest was directed at the effects of function copy number of DAZL on fertility, whereby the Het females have significantly larger litter sizes than their DAZL Wt counterparts. To address the phenotypic differences directly, identifying how the molecular function of DAZL affects oocyte gene expression was investigated (Chapter 3). In addition a novel approach of identifying *in silico* targets of DAZL was employed, exploiting one of the putative DAZL consensus sequences previously identified to be essential for DAZL RNA binding (Chapter 4) (Venables et al., 2001). The RNA binding properties of DAZL were additionally investigated by attempting to identify *in vivo* targets within the ovary by immunprecipitation (Chapter 5) (Reynolds et al., 2005; 2007). Results from the *in silico* trawl (Chapter 4) provided a plethora of potential targets with one candidate, PDCD4, selected for further analysis (Chapter 6). Consequently, from investigating plausible actions of PDCD4 within the ovary and as a DAZL mRNA target, a novel mechanism of programmed cell death was identified in the mouse CL (Chapter 6). In addition to the direct methods explored, DAZL gene function was investigated indirectly by means of *in vitro* follicle culture. It had been previously shown (Elaine Watson, PhD 2007) that, *in vitro,* follicles from DAZL Het mice showed a greater sensitivity to FSH, accelerating in growth and reaching significantly larger sizes than their Wt counterparts. To address these preliminary results further, additional *in vitro* studies were performed to investigate the role of DAZL on FSH sensitivity and determine if any molecular control of

follicle development, and potential bi-directional control of follicle maturation, were significantly altered by investigating gene regulation (Chapter 7).

Evidence from the studies performed on oocyte gene assessment concluded that DAZL is probably having a regulatory effect on several known oocyte-expressed genes that may be essential components for oocyte development. The reduction in mRNA of 10 candidate oocyte genes in d21 oocyte pools, accompanying the decreased functional DAZL copy number in Hets, suggests that the function of DAZL protein may be limiting the direct translation of these identified genes, although reduced DAZL protein formation resulting in translation efficiency has not been investigated. Of selected interest were Oosp1 and H1foo as the mRNA expression of these two oocyte factors were also significantly down-regulated in Het ovaries at d10. Furthermore, using d21 ovaries subsequent analysis of follicles which had been pooled regarding their size and potential developmental stage, showed that the expression levels of these two oocyte specific genes remained different between Het and Wt. However, in contrast to d10, mRNA expression was up-regulated in the Het compared to the Wt but only in follicles of 180μm but not in smaller follicles. Interestingly, Oosp1 has an unknown function (Yan et al., 2001; Choi et al., 2007), but has been demonstrated to be expressed in follicles beyond the primordial stage whereas, H1foo has been implicated in earlier selection processes from primordial to primary stage of development (Tanaka et al., 2005). In addition Nobox, Figla and ZP3, all associated with early primordial follicle formation and oocyte development, were down-regulated in the Het compared to the Wt follicles in the d21 oocyte pools (Liang et al., 1997; Soyal et al., 2000; Suzumori et al., 2002). The down-regulation in expression of both ZP3 and Figla mRNA is consistent with the role of Figla as the transcriptional regulator of ZP3 (Liang et al., 1997; Soyal et al., 2000). Intriguingly, both ZP3 and Cd9, a further candidate down-regulated in the Het oocytes are both associated with fertilisation capacity (Le Naour et al., 2000). However, the reduction in expression does not appear to have any detrimental effects on the fertilisation capacity of oocytes in Het mice *in vivo*. Moreover, DAZL affected the transcription of Zar1, Stella and H1foo, three critical early embryogenesis genes (Wu et al., 2003; Payer et al., 2003; Tanaka et al., 2005).

Collectively these findings suggest a possible developmental role for DAZL either directly or indirectly regulating a subset of genes preferentially expressed in postnatal oocytes, some of which have been shown to play essential roles in oogenesis. Furthermore, these findings also suggest that DAZL may be not only functioning near the top of a signalling cascade regulating genes necessary for oocyte and early follicle development but possibly plays a further role in early embryo development. It can be concluded that DAZL could have either pre-programmed the oocyte with subsequent regulatory effects on growth and sensitivity or is functionally active at different stages of oocyte development. But how these factors may be directly or indirectly associated with DAZL function remain to be fully elucidated.

Previous attempts to identify DAZL RNA binding targets remained inconclusive, with very little evidence to suggest a direct action within the ovary in addition to limited literature on the effects of DAZL mutation of female fertility. The studies in this thesis were the first to attempt to isolate endogenous DAZL protein: RNA complexes directly using mouse oocytes and ovary preparations rather than reconstituting those interactions using recombinant proteins or RNA libraries. To address the binding specificity of DAZL *in vivo* and to investigate direct interactions further, the previously used CLIP technique was employed. This technique has already been used to identify *in vivo* interaction of DAZL within the testis (Reynolds et al., 2005, 2007). We present evidence which supports previously published data that DAZL protein binds to and regulates the translation of MVH within this testis, an important factor in spermatogenesis and oogenesis. However the CLIP was problematic when transferred to the oocyte, at least with the numbers of oocytes used in the present studies. In light of these studies it became apparent that the abundance of DAZL protein within the ovary was such that is was undetectable by Western Blot analysis, and subsequently the CLIP technique as it stands in unsuitable for the use in oocytes. Collectively, the results highlight the difficulties present when working with limited material such as the oocyte and until such a time that the sensitivity of the CLIP technique can be optimised and applied to oocytes these studies to identify *in vivo* DAZL target remain inconclusive. Interestingly these studies did highlight the possibility of a similar DAZL protein being detected within different tissues used

as controls during these studies, including the liver, kidney and heart all of which now require further investigation before conclusions can be drawn.

To further explore DAZL mRNA targets within oocytes in an attempt to further identify if transcription regulation is altered between the Wt and Het, a published consensus sequence (Venables et al., 2001) was used to perform an *in silico* trawl of the mouse genome. The results of this analysis showed that this consensus sequence was highly expressed in many sequences which question the feasibility of it being the only requirement for DAZL binding and subsequent function. The abundance of potential targets additionally highlighted that bioinformatics can only be used as a biological aid and not as a tool for definite answers. Potential targets were identified and selected based on oocyte-expressed genes with suspected functional involvement in apoptosis, cell proliferation or steroidogeneis. However, until such time that direct *in vivo* interactions between oocyte DAZL and target mechanisms these results remain unexplored.

In light of the *in silico* trawl one potential candidate, PDCD4, was selected for further investigation and now an unforeseen role for this protein within the ovary has emerged. We have now determined that PDCD4 may play a fundamental role in regulating programmed cell death within the CL during functional and structural luteolysis. In support of a pro-apoptotic function of PDCD4 in the ovary (Shibahara et al., 1995), in addition to the evidence of protein localisation and translocation from the cytoplasm to the nucleus, a similar pattern of protein expression was observed at luteolysis as previously shown in other cell types undergoing programmed cell death (Schlichter et al., 2001a, b; Bohm et al., 2003; Yang et al., 2003). Through a series of experiments examining induced follicle atresia by FSH withdrawal, induced luteinisation using therapeutic agents of cloprostenol and bromocriptine, and a natural model of luteolysis the pro-estrous mouse, it can be concluded that the localisation of PDCD4 is associated with CL remodelling and becomes nuclear on induced luteolysis in conjunction with an increased abundance in cytoplasmic expression.

Adopting an indirect approach to identify molecular alterations of DAZL function on the follicle unit, which may explain the phenomenon of increased Het litter size, provided evidence that bi-directional control of the granulosa cells may be a further target of DAZL action. Culture systems have provided a wealth of information in association with intra-ovarian, intra-follicular and cross talk communication between follicle in recent years (Spears et al., 1996, 2002; Baker et al., 2001). There was support from previous studies that demonstrated that the Het follicles are more sensitive to FSH *in vitro* (Elaine Watson, PhD 2007). It has now been demonstrated that molecular control of Het follicles is altered compared to the Wt, most notably with reference to the FSHR up-regulation at high FSH culture concentrations, thus, further supporting the proposed hypothesis that the threshold sensitivity of the Het follicles is comparatively altered. The up-regulation of aromatase mRNA in Het follicles suggests advancement in follicle development although there was no difference in LHR mRNA expression. Furthermore, we demonstrated that the relationship between the inhibin βB and inhibin βA subunit expression was altered, suggests that there are fundamental differences in the production of activin and inhibin, with preference for inhibin A production at reduced FSH levels in the Het but inhibin B in the Wt. These findings are suggestive of the advanced stage of follicle development after culture. However, previous results from plasma from d21 mice show that Hets have an increase in inhibin B plasma levels compared to Wt (McNeilly et al., 2000), but these result represent the production from the entire ovary and the two cannot be directly related. These findings emphasise the potential specific role for inhibin subunits in follicle progression and FSH sensitivity. In conclusion, these indirect investigations into the molecular control of follicle development mediated by DAZL functional expression not only suggest that FSH may be playing a modulating role, possibly by regulating its own receptor, but that other regulatory factors are involved. Recruiting members of the TGFβ superfamily into the regulatory control of DAZL follicle development suggests that advanced Het follicles transfer their preference to inhibin A, which has been additionally related to advancement of follicle growth (Knight and Glister, 2003). Furthermore this may be directly related to the increased sensitivity of these Het follicles to FSH and hence decreased levels of follicle atresia. Bearing in mind that the pressure of the ovarian

environment is removed, follicle regulation may be subjected to adaptive modulation *in vitro,* and *in vivo* analysis is now required to address the physiological relevance of these molecular findings.

The assessment of molecular control in the current thesis has been principally directed at the gonadotrophin-dependent stage of follicular development where FSH and LH-signalling pathways play obligatory roles. *In vivo* the increase in plasma FSH during luteo-follicular transition is the basis for follicle selection, with the most sensitive follicle responding first to threshold concentration. Only FSH has been subjected to scrutiny on the sensitivity in the current studies. As previously mentioned, in the gonadotrophin-dependent phase of follicular development, LH also appears to act within a critical minimal threshold and ceiling level for the normal functions of the follicle unit (Palermo, 2007). LH has not been investigated and the roles of LH between Wt and Het follicle development may additionally provide evidence into the developmental differences which are beginning to emerge.

Although not studied in depth, there appears to be no difference in oocyte exhaustion and premature ovarian failure *in vivo* between the Wt and Het (unpublished, Judy McNeilly) suggesting that follicle atresia is also reduced in the cohort of recruited follicles, leading to the overall increased litter sizes. Therefore the results detailed in this thesis showing that Het follicles are less susceptible to atresia *in vitro* regardless of the FSH concentration, supporting that of the physiological observation. Furthermore, it could be argued that the observed increase in Het litter size could additionally occur as a result of increased survival of embryos rather than an increase in ovulation rate and a decrease in atresia rate. However, during preliminary studies investigating the Het phenotype, the number of implantation sites and CL present were directly correlated to the number of embryos present in both Wt and Het ruling out the aforementioned possibility of increased embryo survival (unpublished, Judy McNeilly).

The last decade has witnessed considerable advances in both our understanding of follicular development and atresia, and the tools available to further manipulate and

investigate these processes are becoming greatly exploited with particular reference to transgenic murine models. In addition follicle culture is now a widely used technique which allows one to manipulate the expression of genes of interest and monitor local regulatory events at different stages of development and in a more physiological environment. Together these permit increasingly comprehensive studies and both have been employed and exploited in the current studies. Acquiring an improved understanding of intra-ovarian interaction between follicles of the DAZL transgenic model may help to determine how this genotype recruits follicles for initial development and subsequent ovulation, under reduced FSH thresholds and altered molecular control. Moreover, understanding this may subsequently be a useful application for oocyte selection and further aiding reproductive technologies in human fertility, possibly using DAZL as a biomarker for oocyte selection with reduced DAZL being potentially better for survival.

The many regulatory components related to the processes of folliculogenesis present persisting challenges and when one pathway is thought to be complete another emerges. The importance and timing of follicle development and control are sensitive issues and clearly malfunction results in death. An accurate balance between survival and atresia remains fundamental to healthy viable oocytes with survival factors promoting follicle growth and protecting cells from apoptosis. Absence of survival factor contributes to activation of the endogenous apoptotic pathways leading to follicle atresia. A generalisation regarding DAZL is that in the complete absence, germ cell apoptosis occurs, but Het expression increases survival over and above that of the Wt. Actions are beginning to emerge that DAZL function may be perturbing the system aiding selection criteria and fundamentally altering the sensitivity of the FSH control system.

Finally, from the studies performed in this thesis it is possible that DAZL is regulating follicle selection and subsequent growth directly via oocyte associated mechanisms as demonstrated by oocyte gene expression differences. These in turn may be indirectly playing critical roles in the intra-ovarian bi-directional communication, regulating FSH sensitivity and having an effect on oocyte

maturation and granulosa cell proliferation and differentiation including members of the TGFβ family. Furthermore, understanding how DAZL regulates target mRNAs in its direct function as an RNA binding protein will provide further information about how target gene function relates to the mRNA difference observed within this comparative study at the oocyte, follicle and endocrine level.

Direct and indirect mechanisms of investigation have certainly highlighted that there are alterations in the control systems between the Wt and Het DAZL phenotypes. The possible roles for DAZL in fetal life, predetermining the molecular requirement of subsequent development and in post-translational programming of oogenesis, folliculogenesis at the time of gonadotrophin responsiveness, steroidogenesis, and overall sensitivity of the HPG axis have been accentuated. Understanding the possibility that DAZL may be involved in multiple developmental functions regulating events associated with different targets and transcription mechanism has implications for therapeutic management. Together, the studies in this thesis support the hypothesis that DAZL is essential and critical for germ cell function with the suggestion that the oocyte subsequently alters the sensitivity of follicles to component parts of the HPG. Long term studies are now need to be designed to observe regulation differences between the DAZL Het and Wt from fetal life until cessation of fertility, to understand the developmental competence of follicle growth, the endocrinology, and steroidogenic capacity. Ultimately, the better the understanding of the transcriptional control in oogenesis and subsequent folliculogenesis within the DAZL mouse model, the more rational our approach will be to modulate and assess human fertility problems.

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Appendix 1

Appendix Table: *Oligonucleotide primer sequences used for PCR (A) and Real-Time PCR (B).*

A: PCR

Primer	Forward Sequence	Reverse Sequence
Atm	gCTCTCCCATATCCTgTCTgTC	gCTgCCCTTACTCAACTCTTC
BMP15	CAATgACACCgATgACAgA	gAgCAATgATCCAgTgATCC
BMP6	TTCTTCAAggTgAgCgAggT	TAgTTggCAgCgTAgCCTTT
Cd9	CggTCAAAggAggTAgCAAg	TCACCAAgAggAACCCgAAg
CDC2	TgAAAgCgAggAAgAAggAg	CCCTggAggATTTggTgTAAg
CDC25c	CCTCTgACTTCTCCTCTgTg	gACTCTTCCTCCTCCATCTg
c-kit	ATCCCgACTTTgTCAgATgg	AAggCCAACCAggAAAgTT
Cx37	TCAAAggCACCTCAgAgAC	AATAggggAggCTCgAAAg
Cyclin B1	CCTCACAAAgCACATgACTg	TCgACAACTTCCgTTAgCC
DAZAP1	gAAATATCgACCCgAAgCC	gTCCACTgATTgTTCgTCC
DAZAP2	TCCTCAgACCTTACATCTTCC	CATCATACCCTCCTTCCACC
Dazl	TCCAAATgCTgAgACTTACATg	gTCTgTCTgCTTCggTCCAC
Dmc1	CCAgATgTTgTCACgACTCC	ACCAATTCCCCACCTACTCC
DNMT1	TTACgACgAAgCCAAggAC	ACAgACCTCACAgACACCAC
DNMT2	gggACAggAAACATCAACAAg	ggTggCAAATCAggAAgAg
DNMT3l	ggACCTCAgAgAggATCAATg	AgAACCAgAACCgCTTTCC
Figla	ACAgAgCAggAAgCCCAgTA	ACTCgCACAgCTggTAggTT
Fragilis	CTTCTCAAgCCTTCATCACC	TTCCgATCCCTAgACTTCAC
Gapdh	ACCACAgTCCATgCCATCAC	TCCACCACCCTCTTgCTgTA
GDF9	ATCTgATAggCgAggTgAgAC	CACAAggTCACACATACAgg
Gpiap1	AAgATggAAggAgTggAgTg	CAgAAgAgCCAATCAAAgCC
H1foo	TggAgAAAgggCAgAAgAgg	TTTTggCgAgggAAgCAAC
mEomes	gCAgggCAATAAgATgTACg	CTgAgTCTTggAAggTTCA
Msh4	gAgCTgCATCCAgTTTTCC	TTgCCATTCCTATCTCgCC

Msh5	CAAACAggATgAggCTATgAC	AAgggAATAATggAggAgAgg
MSY2	CATCAACAggAATgACACCAAg	TACgTCgATTAggggCATAgC
MVH	AggAggTgAAAgCAgTgATAg	ATgAgCCAAAATAggCAAgAg
Nobox	CTgCTgCACTggAgACAgAg	CTTCAgCgATTCTgCCTTTC
Nr6a1	TgTTTgCTCTgCTTTgCC	ATCACCTCCATCCCTTCATC
Oas1d	AACTCCTTCCTgACACCACC	CCTCAATgAACACTACCTgTCC
Oct 4	AgCACgAgTggAAAgCAACTC	CAgATggTggTCTggCTgAAC
Oosp1	TgTgAAgCAgAAAgTAgAggAg	ggAgAAATggAAAACCgAgAAg
PABPC1	gAgACCAgCTTCCTCACAgg	ACCTTgCACATgAACAgCAg
PABPC2	AAATgAACgggATgCTTCTg	CTTTCgATTTCCCACCTTCA
Pdcd4	gCTAgAATCAATgCAAAAgCC	TCCACCTCTTCCACATCATAC
Pkd2l2	gCTATCCCgACTTTTACTTCC	ACCCAAAAACCAgAATCCC
Plat	gCTgAgTgCATCAACTggAA	gCCACggTAAgTCACACCTT
Pumillio2	gCAgCAACCAAgCACTAAC	AACTgCCAACTgATgAgCC
Smac/Diablo	gCCTCAgTCTCTCAgTAACg	TgCCACACCTCATCTTCCTC
Stella	ATACagAcgTCCTACAACCAg	CATCTgAATggCTCACTgTC
Sycp3	CCgCTgAgCAAACATCTAAAg	gCATATTCTgTACTTCACCTCC
wee1	CCTCAATCCCAAATgCTgTC	TgCCACTgCTCTCCATTTC
Zarl	gCCgAgTgTgTgAgAAATC	AAgggAAgAggTgAAgAggg
Zp1	AggATTgCCACggATAAgAC	TTgAAAggACACCCATATgAC
Zp2	CATACCCAgACCAATCCTACC	CAgTTCATATTCACAgCCATCC
Zp3	ACTCCTCCCCCTCTCACTTC	AATCACAgATgTCAgCATCAC

B: Real Time PCR

Primer	Forward Sequence	Reverse Sequence
Dazl	gAAggCAAAATCAtgCCAAACAC	CTTCTgCACATCCACgTCATTA
Gapdh	gACATCAagAaggTggTgAagC	gTCCACCACCCTgTTgCTgTAg
MVH	CCACCggCAATTTTgACTTTTg	AAAgCTgCAgTCTTCCCAgAC
Pum2	gCAgCAACCAAgCACTAAC	AACTgCCAACTgATgAgCC